Proximate and ultimate factors influencing reproductive skew in the ant species *Leptothorax acervorum* (Hymenoptera: Formicidae)

DISSERTATION ZUR ERLANGUNG DES
DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN
DER UNIVERSITÄT REGENSBURG

vorgelegt von
Jürgen Trettin

aus
Jena

im Jahr
2015

Das Promotionsgesuch wurde eingereicht am:

31.03.2015

Die Arbeit wurde angeleitet von:

Prof. Dr. Jürgen Heinze

Unterschrift:

To Ivana

&

My Parents

ACKNOWLEGMENTS

I would particularly like to thank...

Prof. Dr. Jürgen Heinze,

... for inviting me to work in his lab and for proposing me the thesis topic

... for advice and support whenever needed

Prof. Dr. Alfred Buschinger,

... for his help in the field, his advices and 'histories' of myrmecology

Prof. Dr. Christoph Oberprieler, PD Dr. Christoph Schubart, Dr. Carsten Löser, Dr. Alireza Keikhosravi, Shobit Agrawal and Nicolas Thiercelin,

... for helpful discussions and suggestions on phylogeography.

Dr. Bartosz Walter,

... for the practical introduction to 'measuring behavior'

Stephanie Leopold, Andi Sailer, Monika Haubner and Thomas Seyferth,

... for 'enduring me patiently' as co-supervisor of their theses (Bachelor or Zulassungsarbeit)

Dr. Ivana Miranda da Silva, Andi Trindl, Masaki Suefuji and many students,

... for advice and help during my laboratory work

Dr. Simon Tragust, Dr. Abel Bernadou and the members of the 'horse group'

... for helpful discussions and advice on statistical issues

Marion Füßl, Monika Haubner, Sabine Hutschenreuther, Peter Koller, Richard Landstorfer, Stephanie Leopold, Doris Rothgänger, Gudrun Schneider, Ivana Miranda da Silva, Thomas Seyferth, Christiane Wanke, many colleagues and students,

... for assistance during the many field trips for ant sampling

Shobit Agrawal, Dr. Cátia Bartilotti, Alireza 'the boss' Keikhosravi, Carsten Löser, Gudrun Schneider and Nicolas Thiercelin,

... for all kinds of support, including fruitful discussions and friendship

Grete & Peter Trettin,

... for their constant support, patience and love

Die Deutsche Forschungsgemeinschaft (He 1623/25)

... for the financial support

... and finally all members over the years from the Heinze lab!

Declaration of thesis's composition and author's contribution

CHAPTER 1 - Queen dominance and worker policing control reproduction in a threatened ant

Jürgen Trettin, Monika Haubner, Alfred Buschinger, Jürgen Heinze

Published in: BMC Ecology (2011) 11: 21

Author contributions

AB and JH devised the study and took part in field work; JT did most of the collection and, together with MH, the genetic and behavioral studies and analyzed the data. JT and JH wrote the paper. All authors read and approved the final manuscript.

CHAPTER 2 - Behavioral Plasticity in Ant Queens: Environmental Manipulation Induces Aggression among Normally Peaceful Queens in the Socially Polymorphic Ant *Leptothorax acervorum*

Jürgen Trettin, Thomas Seyferth, Jürgen Heinze

Published in: PLoS ONE (2014) 9(4): e95153. doi:10.1371/journal.pone.0095153

Author Contributions

Conceived and designed the experiments: JH, JT. Performed the experiments: TS, JT. Analyzed the data: JT, TS. Wrote the paper: JT, JH.

TABLE OF CONTENTS

SUMMARY

In animal societies beside cooperation also conflict among members can arise. Normally, these conflicts emerge among individuals over their share of group's reproductive output, i. e., reproductive skew. In social hymenoptera, additional conflict over resource allocation can arise between members of different castes. Herein this thesis we focus on the first type of conflict, by studying the proximate and ultimate factors of variation in reproductive skew in the ant *Leptothorax acervorum* (Hymenoptera: Formicidae). Over large parts of its Holarctic range, the species shows a low skew colony structure (i. e., one or more breeding queens per colony) while populations from its range margin usually express a high skew colony phenotype (i. e., only one breeding queen per colony). To clarify whether queens and/or worker are involved in the establishment of high skew we observed queen and worker behavior in colonies from central Spain. Among others, we show that aggression among queens leads to the formation of dominance hierarchies, in which only the top-ranking queen becomes fertile (**chapter 1**). Are queens able to react flexibly to changes in reproductive skew, as assumed by skew theory? To answer this question, we experimentally manipulated socio-environmental factors to investigate whether queen-queen aggression can be evoked in low skew colonies. Our results show that queens are able to react to changes in skew in behavior rather than evolutionary time (**chapter 2**). In addition, we performed population genetic and phylogeographic analyses to study the ultimate conditions underlying variation in *L. acervorum's* social organization (**chapter 3**). Finally, we conducted a reciprocal transplantation experiment to investigate whether variation in skew can be affected by its social environment or, alternatively, has a genetic basis only (**chapter 4**).

GENERAL INTRODUCTION

In group living animals not only cooperation but also conflict among group members can emerge. Usually, these conflicts in groups arise over share of reproduction among its members, as genetically non-identical members may have divergent interests about their share of group's reproductive output (e. g. Keller & Reeve 1994, Hager & Jones 2009 and contributions therein, Port & Kappeler 2010). Moreover, in animal societies with unusual genetic family structures (e. g. social Hymenoptera), additional social conflicts over the allocation of resources towards one offspring sex or female larvae's development into sexuals or workers can emerge between individuals of different or same caste (see Heinze 2004, 2010, Ratnieks et al. 2006, Strassmann & Queller 2007 for a detailed discussion). Normally, efficient mechanisms of conflict resolution such as punishment, policing and dominance help to regulate egg laying and development in societies in which all females are morphologically identical and potentially capable of reproducing (Monnin & Ratnieks 2001, Ratnieks et al. 2006). In species with a clear queen-worker diphenism, workers normally refrain from laying eggs in response to the odor of a fertile queen (Bourke 1988, Hammond & Keller 2004), presumably because they otherwise risk to be attacked by their nestmates.

Herein, we focus on the first type of social conflict: the partitioning of reproduction among totipotent members of an animal society, i. e. reproductive skew (Vehrencamp 1983, Reeve & Keller 2001, Port & Kappeler 2010). High skew emerges if one or a few individuals dominate reproduction in a group (e.g. in eusocial insects where normally only one female, the queen, dominates reproduction), while low skew exists in groups where reproduction is

equally shared among members (e. g. in banded mongooses [*Mungos mungo*], Sherman et al. 1995, Gilchrist et al. 2004).

Reproductive or optimal skew theory provides an explanatory framework for how reproductive skew is adjusted and thus reproductive conflicts are resolved among group members (e. g. Vehrencamp 1983, Reeve & Keller 2001, Ratnieks et al. 2006, Nonacs & Hager 2011, Trubenová & Hager 2012). The theory includes several optimal skew models, each one with its own assumptions and predictions that can be distinguished into two main classes of models. Briefly, in 'transactional models' reproductive skew is the outcome of reproductive transactions between dominant and subordinate group members, mediated either by the subordinates' threat to leave the group (concession models) or by the dominants' threat to expel them from the group (restraint models). In contrast, 'compromise models' assume that reproductive skew is the outcome of a competition over reproduction between group members, which intensity is mediated only by the costs it imposes on group productivity (see Johnstone 2000, Nonacs & Hager 2011, Reeve & Shen 2013, Kappeler et al. 2013 for detailed reviews). Key parameters in most skew models that determine the degree of reproductive skew within groups are the genetic relatedness among its members, their respective fighting strength, the impact on group productivity and ecological constraints on breeding independently (e. g. Keller & Reeve 1994, Johnstone 2000, Nonacs & Hager 2011).

However, despite the initial success, advance and proliferation of skew models empirical evaluating and testing of their assumptions and predictions have lagged behind that theoretical development over the last two decades (e. g. Magrath & Heinsohn 2000, Kokko 2003, Nonacs & Hager 2011), even leading to strong resistance against more model variants (Reeve & Shen 2013). For instance, skew models assume that group members adaptively adjust skew to their current situation. Alternatively, group members might be genetically predisposed to either fight or tolerate a rival reproductive, i. e. reproductive skew might be an evolutionary (genetically constraint) rather than a behavioral response to environmental change (Kokko 2003).

In addition, an adjustment of skew in behavioral time might be limited by further types of mutually non-exclusive constraints, such as developmental, social or ecological constraints (Kappeler et al. 2013). Indeed, whether queens are capable of flexibly reacting to changed environmental conditions (Kokko 2003) has rarely been investigated.

Nevertheless, while most studies of reproductive skew in mammalian societies have found support for compromise models (Port & Kappeler 2010, Kappeler et al. 2013), studies of skew in social insect societies usually found support for the transactional types of model (e. g. Reeve & Keller 2001). In particular, the latter models predict that high reproductive skew should be evolutionary stable when the costs of dispersal (and hence becoming reproductive away from the established nest) and relatedness among nestmates are high (e. g. Keller & Reeve 1994). In addition, models predict that higher levels of skew should be associated with higher levels of within-group aggression and vice versa (e. g. Bourke & Heinze 1994, Reeve & Keller 2001). Support for both predictions can be found in Formicoxine ants, in which high skew species are indeed specialists of patchy habitats and queen-queen aggression is involved in the establishment of reproductive hierarchies. In contrast, low skew species living in homogenous habitats such as the extensive boreal forests of Northern hemisphere, don't show any aggressive establishment of hierarchies and finally contribute equally to colony's offspring (Bourke & Heinze 1994, Heinze 2010).

For our studies we chose as a model the Formicoxine ant species *Leptothorax acervorum* (FABRICIUS 1793) for which reproductive skew appears to vary with habitat characteristics. The species is widely distributed over large parts of the northern hemisphere (Francoeur 1983, Seifert 2007, Czechowski 2012) where in extensive coniferous forests its colonies show a low skew social structure (facultative polygyny, Buschinger 1968, Bourke 1991, Heinze et al. 1995a & b). In contrast, the species expresses a high skew colony structure (functional monogyny) in patchy habitats (i. e., on sun exposed slopes in Alaska, in light clearings in

Hokkaido, and in mountainous areas in central Spain; Ito 1990, Heinze & Ortius 1991, Felke & Buschinger 1999, Gill et al. 2009).

Aims and parts of the present thesis

Functional monogyny in high skew populations of *L. acervorum* appears to be based on queen dominance interactions and fighting in colonies from Alaska (Heinze & Ortius 1991) and Hokkaido (Ito 2005). However, a recent study suggested that queen-queen interactions are rare in the population from central Spain and that instead worker aggression regulates which queen may lay eggs (Gill & Hammond 2011). We therefore observed queen and worker behavior in colonies from the same population during the establishment phase of reproductive skew (**chapter 1**).

Are queens capable of flexibly reacting to changed environmental conditions, as assumed by models of optimal skew (Kokko 2003)? To answer this question we investigated whether queen-queen aggression can be elicited by experimental manipulation of socio-environmental factors in colonies from low skew populations (**chapter 2**).

In **chapter 3** we studied the ultimate conditions underlying variation in reproductive skew by means of population genetic and phylogeographic analysis. Among others, we tested for patterns of variation and differentiation in mitochondrial DNA and 10 nuclear micro-satellite markers between both social forms of *L. acervorum.*

Finally, we conducted a transplantation experiment to investigate whether queens' social environment may affect their behavior towards other queens or, alternatively, whether factors underlying variation in queens' behavior have a genetic basis only, in low and high skew colonies of *L. acervorum* (**chapter 4**).

CHAPTER 1

Queen dominance and worker policing control reproduction in a threatened ant

Jürgen Trettin[1*], Monika Haubner[1], Alfred Buschinger[2], Jürgen Heinze[1]

Address: [1] Biologie I, Universität Regensburg, Universitätsstr. 31, Regensburg, 93053, Germany and [2] Rossbergring 18, Reinheim, 64354, Germany

[*] e-mail: juergen.trettin@ur.de

Phone: +49 9419434264

Fax: +49 941 943 3304

Published in: BMC Ecology (2011) 11: 21

Abstract

Efficient division of reproductive labor is a crucial characteristic of social insects and underlies their ecological and evolutionary success. Despite of the harmonious appearance of insect societies, nestmates may have different interests concerning the partitioning of reproduction among group members. This may lead to conflict about reproductive rights. As yet, few studies have investigated the allocation of reproduction among queens in multi – queen societies ("reproductive skew"). In the ant *Leptothorax acervorum*, reproductive skew varies considerably among populations. While reproduction is quite equally shared among nestmate queens in most populations from boreal Eurasia (low skew), colonies from populations at the edge of the species' range are characterized by "functional monogyny," i.e., high skew. The proximate mechanisms underlying high skew, in particular how workers influence which queen lays eggs, are not well understood. We investigated the behavior of queens and workers in functionally monogynous colonies of *L. acervorum* from two mountain ranges in central Spain. We provide evidence for both queen and worker influence on the outcome of conflict over reproduction in colonies of *L. acervorum* from Spain. The patterns of queen – queen aggression and worker – queen grooming and feeding after hibernation allowed predicting, which queen later began to lay eggs. In contrast, worker aggression towards queens was not clearly associated with a queen's future reproductive success. Queen – queen and worker – queen aggression differed in quality: queens typically engaged in ritualized dominance behavior, such as antennal boxing, while workers also attacked queens by biting and prolonged pulling on their legs and antennae. In several cases, overt worker aggression led to the expulsion of queens from the nest or their death. We conclude that queens of *L. acervorum* from Spain establish rank orders by ritualized dominance interactions, such as antennal boxing. Workers may reinforce these hierarchies by preferentially feeding and grooming high ranking queens and attacking lower ranking queens.

Aggressive worker policing may thus stabilize functional monogyny. Optimal skew models predict that high skew in ants is associated with high dispersal costs. In central Spain, *L. acervorum* is restricted to small patches at higher elevations, which presumably makes dispersal and colony founding difficult. Because of the ecological requirements of *L. acervorum* and the predicted large impact of global change on central Spain, the functionally monogynous populations of this ant must be considered as threatened.

Introduction

Efficient division of reproductive labor is one of the key characteristics of social insects (e.g., honeybees, ants, and wasps) and underlies their enormous evolutionary success. Despite of the harmonious appearance of their societies, who reproduces and who does not is often controversial, and how conflict about reproductive rights is resolved has become focus of both theoretical and empirical research (Ratnieks 1988, Heinze et al. 1994, Heinze 2004, Ratnieks et al. 2006). Punishment, policing, and dominance regulate egg laying in societies in which all females are morphologically identical and potentially capable of reproducing (Monnin & Ratnieks 2001, Ratnieks et al. 2006). In species with a clear queen-worker diphenism, workers normally refrain from laying eggs in response to the odor of a fertile queen (Bourke 1988, Hammond & Keller 2004), presumably because they otherwise risk to be attacked by their nestmates. Worker altruism in insect societies therefore can be considered to be "enforced" at least in part (Wenseleers & Ratnieks 2006).

Comparatively little is known about another type of conflict, the one about the partitioning of reproduction when colonies contain multiple queens. In facultatively polygynous species, egg laying rates and genetic maternity assignments usually suggest a more or less equal contribution of queens to the egg pile and queens do not interact aggressively ("low reproductive skew"; Buschinger 1968, Bourke 1991, 1994, Heinze et al. 1995a & b). Brood from individual queens may differ in its propensity to develop into sexuals, but this is not due to social interactions among queens (Schwander et al. 2010).

High reproductive skew, i.e., a highly unequal partitioning of reproduction, has as yet been described for only a handful of species ("functional monogyny"; Buschinger 1968). For example, in *Leptothorax gredleri* and related species, nestmate queens violently antennate and bite one another and form social hierarchies, in which only the top-ranking queen begins to reproduce (Heinze & Smith 1990, Heinze et al. 1992, Lipski et al. 1992, Ito 2005). At a later

stage, subordinate queens may be attacked and expelled by workers (Heinze & Smith 1990, Heinze et al. 1992, Lipski et al. 1992, Ito 2005). This resembles the elimination of surplus queens in founding associations (Forsyth 1980, Choe & Perlman 1997) and polygynous species (e.g., Lipski et al. 1992).

In accordance with models of optimal skew (Keller & Reeve 1994), functional monogyny is associated with patchy habitat in which solitary nest founding is costly (Bourke & Heinze 1994). In the Holarctic ant *Leptothorax acervorum,* reproductive skew appears to vary with habitat characteristics. *L. acervorum* is widely distributed over large parts of the northern hemisphere (Seifert 2007, Francoeur 1983). Colonies are facultatively polygynous in the extended coniferous forests of Central Europe and Siberia (Buschinger 1968, Bourke 1991, 1994, Heinze et al. 1995a & b), but functionally monogynous where they are only patchily distributed, i.e., on sun-exposed slopes in Alaska, in light clearings in Hokkaido, and at the southern limit of its range in mountainous areas in central Spain (Ito 1990, 2005, Heinze & Ortius 1991, Felke & Buschinger 1999, Gill et al. 2009).

Functional monogyny appears to be based on queen dominance interactions and fighting in colonies from Alaska (Heinze & Ortius 1991) and Hokkaido (Ito 2005). However, a recent study suggested that queen-queen interactions are rare in the population from central Spain and that instead worker aggression regulates which queen may lay eggs (Gill & Hammond 2011). Here, we document that both queen dominance and worker policing contribute to the regulation of reproduction in *L. acervorum* from central Spain.

Results

Details on the origin and composition of colonies used in this study are given in Table 1.1. All queens (n = 35) used in the analysis had sperm in their spermathecae. As expected from previous dissections, in each colony only a single queen had fully active ovaries with elongated ovarioles and corpora lutea. Several other queens had partly elongated ovarioles

showing traces of previous, temporary egg production. However, their ovaries appeared to have reverted to an inactive state (Table 1.2). The colonies SA 03 and SA 88 were observed (Table 1.1) but their queens could not be dissected. Consequently, both colonies were removed from statistical analysis.

Casual observations had already indicated queen-queen aggression directly after collecting in fall. More detailed studies after artificial hibernation corroborated this result: aggressive interactions among queens occurred in 11 out of 12 studied colonies from S^{ra} de Albarracin and S^{ra} de Gúdar (Figure 1.1). In all colonies, we in addition observed worker aggression towards queens. In total, queens received 53% of attacks from other queens (median and quartiles per queen 0.12, 0.0, 0.9 attacks per hour). Workers were responsible for 47% of the attacks toward queens (median and quartiles per queen: 0.36, 0.12, 1.16 attacks per hour). In addition to the antagonistic behavior, we also observed sociopositive interactions (grooming and feeding). The observed level of grooming and trophallaxis received by queens ranged from zero to 2.75 events h^{-1} (median, quartiles per queen: 0.7, 0.44, 1.33 acts per hour). The quality of queen-queen and worker-queen antagonism differed considerably. Queens were significantly more often pulled by workers than by other queens (Figure 1.2, Mann-Whitney U test: $U = 16$, $N_1 = N_2 = 10$, $P = 0.008$). We found a similar trend for differences in biting (Figure 1.2, $U = 27.5$, $P = 0.093$). In contrast, there was no significant difference between queens and workers in the frequency of antennal boxing (Figure 1.2, $U = 43$, $P = 0.61$) and mandible threats (Figure 1.2, $U = 43$, $P = 0.63$). In numerous instances we observed several workers simultaneously pulling on the antennae or legs of a queen. This severe pulling occasionally led to expulsion of queens and to the death of three queens from three colonies.

Statistical analysis showed that, overall colonies, future reproductive status of queens was not reliably predicted by worker-queen aggression (logistic regression: likelihood- ratio-Chi^2 = 0.05, d. f. = 1, $P = 0.83$, 31 queens), but instead by the frequency of sociopositive acts from

workers to queens (logistic regression: likelihood- ratio-Chi^2 = 11.42, d. f. = 1, $P < 0.001$, 35 queens). Furthermore, the individual average dominance index (ADI, Hemelrijk et al. 2005) of each queen calculated from queen-queen aggression predicted the future reproductive status of a queen (likelihood-ratio-Chi^2 = 4.50, d. f. = 1, $P = 0.034$, 29 queens). These results are corroborated by Scheirer-Ray-Hare tests: worker-queen aggression was not associated with the queens' future reproductive status (H = 0.06, d. f. = 1, $P = 0.81$) in contrast to worker-queen grooming and trophallaxis (H = 5.61, d. f. = 1, $P = 0.018$) and, although at only marginal significance, ADI (H = 3.03, d. f. = 1, $P = 0.08$). Both queen aggression (8 out of 560 attacks) and worker aggression (16 out of 572 attacks) led to the expulsion of subordinate queens.

Across all five microsatellite loci, average relatedness of nestmate workers from 10 colonies from S^{ra} de Albarracin was 0.67 ± SE 0.06. A linkage analysis revealed significant linkage disequilibrium between the loci LXAGA1 and Myrt3 ($P = 0.005$). Therefore, we repeated the relatedness analysis without Myrt3 and obtained a relatedness coefficient of 0.66 ± 0.05. Both values are not significantly less than the value expected for full sisters (0.75; t = -1.489, $P = 0.17$ vs. t = -1.592, $P = 0.15$). The inbreeding coefficient (F = 0.10 ± 0.08) was not significantly different from zero (t = 1.25, $P > 0.2$).

Discussion

Our study provides evidence for the regular occurrence of aggressive interactions among queens in functionally monogynous colonies of the ant *L. acervorum* from central Spain. Queen-queen aggression leads to the establishment of rank orders, in which the top-ranking queen becomes fertile. Queen dominance behavior, together with additional, unevenly distributed aggressive or sociopositive actions of workers, proximately underlie high reproductive skew and lead to functional monogyny.

Both, the frequency of queen-queen aggression and of grooming and feeding behavior of queens by workers predicted which queen later started to lay eggs. Future reproductive queens typically showed a high level of aggression towards other queens and were frequently groomed and fed by workers. In contrast to queen antagonism, worker aggression towards queens was not associated with future reproductive status. Worker aggression was considerably more overtly aggressive than queen aggression.

Concerning the mechanisms underlying functional monogyny, *L. acervorum* from central Spain thus is similar to other functionally monogynous ants. Similar aggressive interactions among mated queens were observed previously in *L. acervorum* from Japan (Ito 2005) and Alaska (Heinze & Ortius 1991), and functionally monogynous *Leptothorax* sp. A, *L. gredleri*, and *Formicoxenus provancheri* (Heinze & Smith 1990, Heinze et al. 1992, Heinze et al. 1993). In all this cases, antagonistic behavior among queens after hibernation contributes to the establishment of dominance hierarchies in which only the highest-ranking individual is reproducing. Queen-queen aggression is commonly complemented by discriminatory treatment of queens by workers. Worker behavior appears to accentuate rank differences, in that workers feed and groom dominant queens more frequently and attack, expel or even kill subordinate queens. For example, subordinate queens of *Leptothorax* sp. A and *L. gredleri* are attacked by workers when reentering the nest after having been expelled by dominant queens (Heinze & Smith 1990, Heinze et al. 1992).

Similarly, both queen-queen and worker – queen aggression have been observed in functionally monogynous colonies of *L. acervorum* from Japan and Alaska (Ito 2005, Heinze & Ortius 1991). It is therefore not surprising that worker aggression occurs in *L. acervorum* from Spain. It serves to prevent surplus queens from becoming reproductive (Lipski et al. 1992) and can thus be considered as policing (e.g., Gill & Hammond 2011, Heinze & d'Ettorre 2009). Workers therefore play an important role in the regulation of reproduction, but presumably only after the queens themselves have established rank orders. As suggested

by Gill and Hammond (2011), workers may act as “agents” of the dominant queen, in that they eliminate rival queens once the hierarchy has formed. Queens of functionally monogynous *L. gredleri* besmear their opponents with Dufour gland secretions, which elicit aggression from workers (Heinze et al. 1992, Heinze et al. 1998). Similar “punishment” is known from the queenless ant *Dinoponera quadriceps* (Monnin et al. 2002) and the fighting males of *Cardiocondyla* (Yamauchi & Kawase 1992). Queen besmearing has not been observed in *L. acervorum* from Spain, but workers might use more subtle signals or chemical differences among queens to discriminate dominant and subordinate queens. Genetic data suggest that at least in *L. gredleri*, queens may seek adoption into an alien colony and usurp the top-position in the hierarchy (J.H., unpublished). Workers appear to support the queen that first becomes reproductive and not necessarily their own mother, similar to the situation in founding associations (Forsyth 1980).

Colonies of *L. acervorum* from Central, Western, and Northern Europe are facultatively polygynous (e.g. Buschinger 1968, Bourke 1991, 1994, Heinze et al. 1995a & b), i.e., the species is functionally monogynous in the periphery of its geographic range. This matches predictions from skew models: marginal areas are suboptimal, and suitable sites for colony founding and nesting are limited. Therefore, such marginal or patchy habitats should favor the development of multiple-queening with less dispersal, higher queen relatedness, higher aggression and higher skew in ant colonies (Keller & Reeve 1994, Bourke & Heinze 1994). In the mountains of central Spain, *L. acervorum* are restricted to humid areas in pine dominated forests at elevations above 1500m (Felke & Buschinger 1999). Though we did not map the location of colonies in detail, colonies appeared to be restricted to relatively small patches surrounded by large, unpopulated areas. Given that the Sierras of central Spain are not much higher than 2000m and expected to be extremely affected by global change (Thuiller et al. 2005), it is likely that such patches will become less and less common. Even though functionally monogynous colonies have been found in more northern mountain ranges (JT

unpublished), the Spanish high-skew populations of *L. acervorum* are probably highly threatened, in contrast to the wide-ranging low-skew populations of boreal Eurasia.

The results of our behavioral study stand in marked contrast to previous observations that worker-queen aggression rather than queen-queen antagonism underlies high reproductive skew (Gill & Hammond 2011). At present we can only speculate about the cause for this discrepancy. First, our study revealed considerable variation in the occurrence of queen-queen aggression among colonies. There may be subtle differences in ecological, behavioral, or genetic properties between the colonies studied by Gill and Hammond (2011) and those in our study. Second, and more importantly, the two studies differ in several critical aspects. The behavioral analysis of Gill and Hammond (2011) apparently did not include antennal boxing, but focused on more violent aggression, which, as we show above, is more commonly exhibited by workers. Furthermore, they subjected the ants to only six weeks of artificial hibernation. This is much shorter than natural winter and also shorter than the standard hibernation time established for laboratory cultures of *Leptothorax* ants by Buschinger (1973). Conditions in S^{ra} de Albarracin and S^{ra} de Gúdar are harsh and temperatures can fall below 0°C during seven months or longer (Felke & Buschinger 1999). *Leptothorax* are active at nest temperatures of 5°C for several weeks after the onset of hibernation (Heinze et al. 1996). Young queens begin to establish hierarchies after mating in late summer and fall (Ortius & Heinze 1999, see also our study) and a period of six weeks is probably too short to obscure rank differences among queens.

Conclusion

Our study documents that aggression among nestmate queens occurs in colonies of two functionally monogynous populations of *L. acervorum* from central Spain. Queen-queen aggression contributes significantly to the formation of reproductive hierarchies among queens and ultimately to the high reproductive skew in these populations.

In addition, we could show that workers influence skew, especially by differentially feeding and grooming queens. In contrast, the frequency of worker-queen aggression was not associated with a queen's future reproductive success, though worker aggression is certainly involved in later eliminating subordinate queens or driving them out of the nest. The intraspecific variation in reproductive skew makes *L. acervorum* a valuable model system to investigate in more detail the proximate and ultimate mechanisms underlying the evolution of alternative reproductive tactics and strategies in social insects.

Methods

Ant collection and cultivation

According to detailed field studies by Felke & Buschinger (1999), *L. acervorum* in central Spain is restricted to pine forests at elevations above 1500m. We therefore focused our study on these previously described collecting sites in the Sierra de Albarracin (September 2008 and May 2009) (Felke & Buschinger 1999) and also identified similar sites at Sierra de Gúdar (May 2009), both province of Teruel, Spain (Table 1.1). Colonies were housed in the laboratory in three-chambered plastic boxes (10cm x 10 cm x 3 cm) with plaster floor using standard methods (Buschinger 1974a). We kept the ants in incubators in near-natural conditions with ten to twelve weeks hibernation (at 12h/12h 5°C/0°C), and thereafter at spring conditions (12h/12h 20°C/10°C) for the duration of the behavioral studies (Buschinger 1973). Meteorological data (Atlas Climático Digital de Aragón) and observations suggest that the ants hibernate for an even longer period in the field. Ants were fed with honey, cockroaches and water twice weekly.

Behavioral observations and ovary dissections

For the behavioral observations, we chose colonies with four to seven queens (Table 1.1). All queens were individually marked with 30 to 88 µm thin metal wires (red enameled, black,

green, violet and copper) tied between alitrunk and petiole, petiole and postpetiole, and / or postpetiole and gaster.

Observations were started three days after marking and carried out under spring conditions (20°C/10°C). Colonies were directly observed in 20 to 60-min sessions each under a binocular microscope by scan sampling every 5 minutes and in addition by opportunistic sampling (Martin & Bateson 2007). We chose to observe interactions directly as one of the most frequent aggressive interactions in ants, rapid bouts of antennal boxing, is not easily detected on video recordings. We noted the occurrence of all interactions involving queens (antennal boxing, mandible opening, biting, pulling, stinging/ smearing, grooming, and trophallaxis, i.e., exchange of liquid food). Overall, we observed the eight colonies for 580 to 1645 min. Rates of behavior were calculated as the frequency of behavior divided by the total length of time a focal colony was observed (hours, Table 1.1) and the number of queens per colony. They are indicated as behavioral events per hours and individual.

After the observation period, we killed the queens by freezing them at -20°C and dissected their ovaries under a binocular microscope to check for ovarian status. Dissections were carried out as described in (Buschinger & Alloway 1978). We noted the presence of maturing oocytes, corpora lutea, and sperm in the spermatheca. Ovarian status was classified following (Heinze et al. 1992).

Statistical analyses

The antagonistic behavior among queens was used to calculate the average dominance index (ADI, Hemelrijk et al. 2005) for each queen per colony. For the analysis of relationships between specific behavioral interactions and the future reproductive state of queens we conducted a logistic regression (reproductive status was binary coded: ‘reproductive’ = 1, ‘non – reproductive’ = 2). We used the Scheirer-Ray-Hare test (Sokal & Rohlf 1995) as an independent method to compare the predictive power between behavioral

interactions and the future reproductive state of queens. All unmated queens and queens with undetermined reproductive state were omitted from analysis. The Mann-Whitney U-test was used to test for differences between queen-queen aggressive behavior and worker-queen aggression. All statistical analyses were carried out in SPSS version 17 and JMP 8.01 (SAS, 2009). Scheirer-Ray-Hare test was performed with EXCEL version 2007.

Genetic analysis

In addition to the behavioral studies we extracted genomic DNA from 119 workers out of ten colonies from one sample site in the Sierra de Albarracin (SA 64, SA 65, SA 66, SA 68, SA 70, SA 74, SA 76, SA 78 & SA 88, 12 workers and SA 61, 11 workers) using a CTAB (Cetyltrimethyl ammonium bromide) protocol (modified after Sambrook & Russell 2001). Eleven to twelve workers per colony were genotyped at five polymorphic microsatellite loci that have previously been shown to be informative in this genus: LXAGA1, LXAGA2, LXAGT1 (Bourke et al. 1997), L18 (Foitzik et al. 1997), and Myrt3 (Evans 1993).

PCR conditions were mainly as previously described (Sambrook & Russell 2001, Bourke et al. 1997, Foitzik et al. 1997) with following annealing temperatures: LXAGA1 at 45°C, LXAGA2 at 50°C, LXAGT1, L18 and Myrt3 at 54°C. Primers were labeled with FAM, HEX, TET fluorescence dyes (Eurofins MWG) and amplification products were analyzed with a capillary sequencer (ABI PRISM 310 Genetic Analyser, Applied Biosystems).

We determined allele length using the software GENESCAN 3.1 (PE Biosystems). Worker genotypes were used to estimate nestmate relatedness (r ± SE by jackknife over colonies; Queller & Goodnight 1989) with RELATEDNESS 4.2. In addition, Fisher's method implemented in GENEPOP 4.0 (Rousset 2008) was used to test for linkage disequilibrium between the five microsatellite loci.

Authors' contributions

AB and JH devised the study and took part in field work; JT did most of the collection and, together with MH, the genetic and behavioral studies and analyzed the data. JT and JH wrote the paper. All authors read and approved the final manuscript.

Acknowledgements

We thank Volker Schmid for help with the statistical analysis in JMP. Supported by Deutsche Forschungsgemeinschaft (He 1623/25).

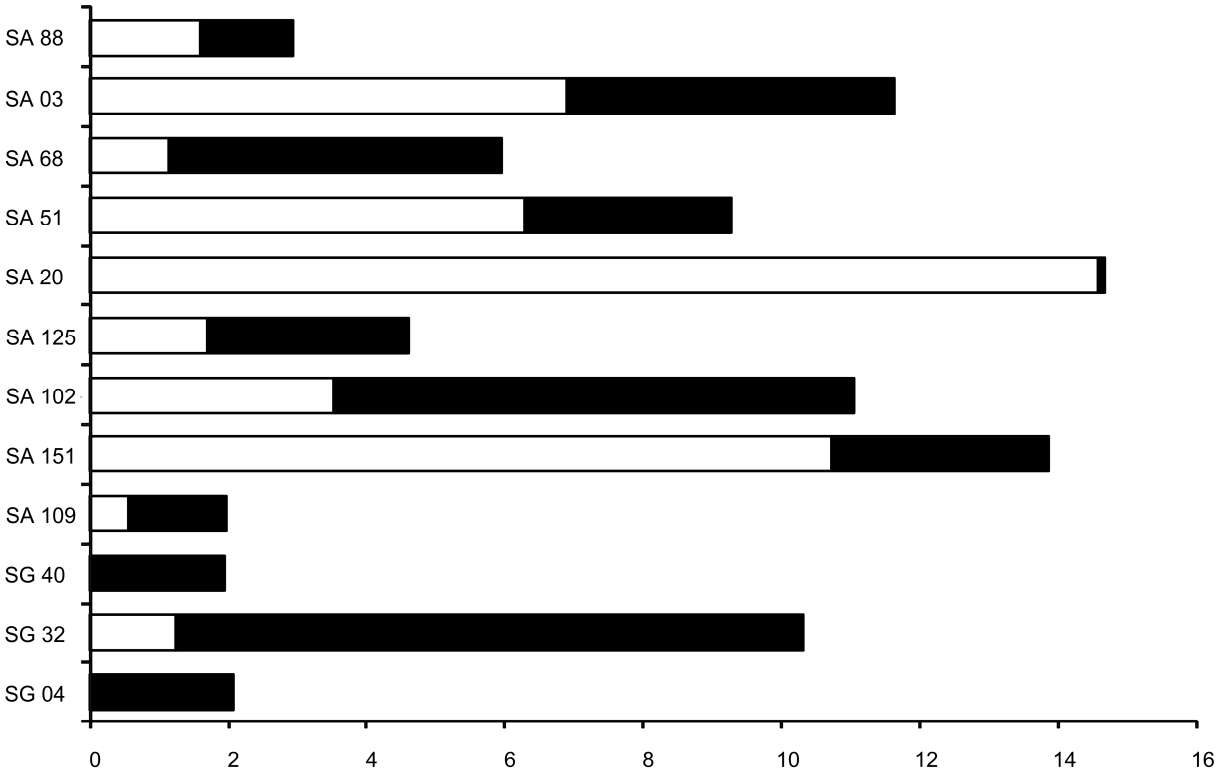

Figure 1.1 Aggression among queens (white bars) and between workers and queens (black bars) in colonies of the ant *Leptothorax acervorum* from central Spain. Aggression is shown in attacks h^{-1}.

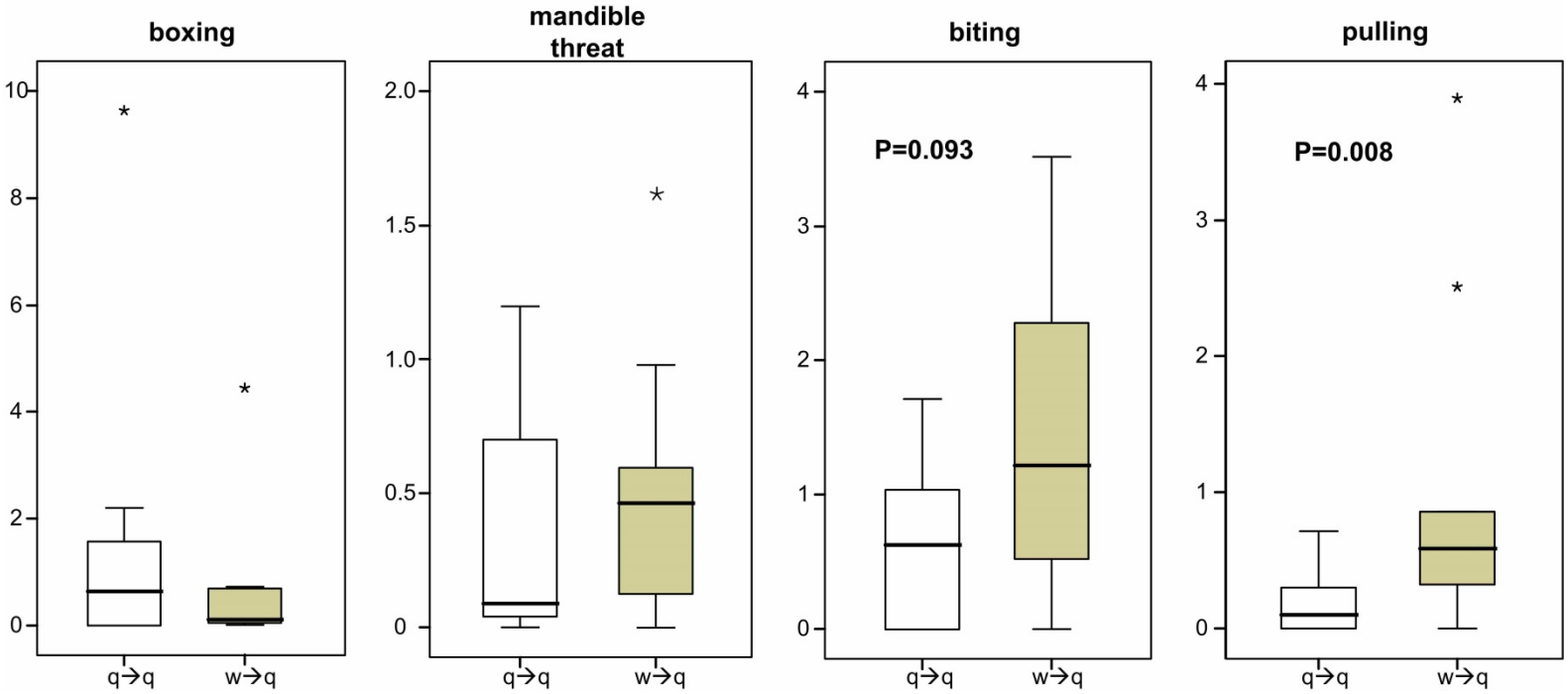

Figure 1.2 Differences in the type of aggressive behavior between workers and queens. Queens show significantly less pulling behavior than workers (N=10 colonies). Worker – queen aggression (w → q) is shown as grey boxes and queen – queen aggression as white boxes. Outliers are indicated as *.

Table 1.1 Location, composition and observation time for each colony.

Site	Colony	Location	Altitude (in m)	No. Queens	No. Workers	Observation time (in hr)
S^{ra} de	SA 03	40.53°, -1.65°	1718	5	30±10	8.4
Albarracin	SA 20	40.50°, -1.59°	1667	4	25	9.7
	SA 51	40.50°, -1.59°	1667	4	30	7.0
	SA 68	40.60°, -1.71°	1683	6	40	8.8
	SA 88	40.60°, -1.71°	1683	5	50±10	7.5
	SA 102	40.50°, -1.59°	1667	6	15	15.9
	SA 109	40.50°, -1.59°	1667	7	20	26.8
	SA 125	40.60°, -1.71°	1683	4	25	10.0
	SA 151	40.53°, -1.64°	1657	6	20	27.9
S^{ra} de	SG 04	40.37°, -0.63°	1959	5	40	15.4
Gúdar	SG 32	40.39°, -0.67°	2014	4	15	24.8
	SG 40	40.39°, -0.64°	1958	5	30	20.4

Table 1.2 Mating and reproductive status of queens for all colonies used in the analysis.

	No. Queens			No. Queens	
Colony	Mated	Unmated	UD	Reproductive	Non-reproductive
SA 20	4	0	0	**2**	2
SA 51	2	0	2	1	3
SA 68	6	0	0	1	5
SA 102	2	3	1	1	5
SA 109	5	1	1	1	6
SA 125	3	1	0	1	3
SA 151	4	2	0	1	5
SG 04	3	1	1	1	4
SG 32	3	1	0	1	3
SG 40	4	1	0	1	4

Data indicated as the number of queens per category and colony. With the exception of colony SA 20, all colonies had one reproductive queen. As colony SA 20 was observed only for a rather short period (9.7 h over 10 days), the reproductive hierarchy, and hence functional monogyny, could not be fully established. The mating status of several queens that were killed before dissection could not be determined (UD).

CHAPTER 2

Behavioral Plasticity in Ant Queens: Environmental Manipulation Induces Aggression among Normally Peaceful Queens in the Socially Polymorphic Ant *Leptothorax acervorum*

Jürgen Trettin*, Thomas Seyferth, Jürgen Heinze

Biologie I, Universität Regensburg, Regensburg, Germany

* e-mail: juergen.trettin@ur.de

Phone: +49 9419434264

Fax: +49 941 943 3304

Published in: PLoS ONE (2014) 9(4): e95153. doi:10.1371/journal.pone.0095153

Abstract

The behavioral traits that shape the structure of animal societies vary considerably among species but appear to be less flexible within species or at least within populations. Populations of the ant *Leptothorax acervorum* differ in how queens interact with other queens. Nestmate queens from extended, homogeneous habitats tolerate each other and contribute quite equally to the offspring of the colony (polygyny: low reproductive skew). In contrast, nestmate queens from patchy habitats establish social hierarchies by biting and antennal boxing, and eventually only the top-ranking queen of the colony lays eggs (functional monogyny: high reproductive skew). Here we investigate whether queen-queen behavior is fixed within populations or whether aggression and high skew can be elicited by manipulation of socio-environmental factors in colonies from low skew populations. An increase of queen / worker ratio and to a lesser extent food limitation elicited queen-queen antagonism in polygynous colonies from Nürnberger Reichswald similar to that underlying social and reproductive hierarchies in high-skew populations from Spain, Japan, and Alaska. In manipulated colonies, queens differed more in ovarian status than in control colonies. This indicates that queens are in principle capable of adapting the magnitude of reproductive skew to environmental changes in behavioral rather than evolutionary time.

Introduction

The organization of animal societies and the underlying behavioral traits vary considerably among species but appear to be remarkably robust within species or at least within populations (e. g., Wilson 1971, Ostfeld 1990, Linklater 2000). For example, the share of individual group members in the offspring produced by the group ("reproductive skew") ranges from equal partitioning among mutually tolerant group members ("low skew") to reproduction being the privilege of only one individual, which dominates all other individuals ("high skew"; Keller & Reeve 1994, Magrath & Heinsohn 2000, Reeve & Keller 2001). Reproductive skew may be affected by ecological constraints on solitary nesting, the relatedness and relative fighting strength of group members, and other factors (see e. g., Keller & Reeve 1994 and Reeve & Ratnieks 1993, Johnstone 2000, Nonacs & Hager 2011 and references therein). Consequently, reproductive skew and the causative behavioral processes typically vary less within than between species or populations (Jamieson 1997, Kutsukake & Nunn 2006).

In most social insects, queens in mature multi-queen societies show little antagonism and contribute equally to the offspring of the group ("polygyny"). High or even maximal skew has been described only from a small number of species with multi-queen colonies, where only one of several inseminated queens monopolizes reproduction (Pardi 1946, Buschinger 1968, Heinze & Smith 1990, Yamauchi et al. 2007). This "functional monogyny" (Buschinger 1968) results from the formation of social rank orders among potential reproductives through overtly aggressive or ritualized dominance behavior (e. g., Pardi 1946, Heinze & Smith 1990, Heinze et al. 1992, Reeve 2000, Yamauchi et al. 2007). The magnitude of reproductive skew and/or the associated behavior of queens in mature or founding colonies appear to be largely fixed within species (Buschinger 1968) or at least within populations (Heinze et al. 1995a, Gill et al. 2009, Helms et al. 2013, Overson et al. 2013). Whether queens are capable of

adaptively reacting to changed environmental conditions, as assumed by models of optimal skew (Kokko 2003), has rarely been investigated.

Here, we examine whether experimental manipulation of colonies from a population with mutually tolerant queens and low skew can elicit queen-queen fighting similar to that underlying reproductive hierarchies in high-skew populations, i. e., whether queens are in principle capable of adjusting their reproductive behavior to changed environmental conditions. Our study species, the Holarctic ant *Leptothorax acervorum* (Fabricius, 1793), is socially polymorphic and the magnitude of reproductive skew varies among populations (Heinze et al. 1995a, Gill et al. 2009). Sexuals of *L. acervorum* mate in late summer and thereafter young, mated queens may seek re-adoption into their natal nests. In low-skew populations, several nestmate queens continue to co-exist peacefully and lay eggs (Bourke 1991, Stille et al. 1991, Heinze et al. 1995a & b, Hammond et al. 2006). In contrast, queens in high-skew populations engage in aggressive interactions and form dominance hierarchies after queen re-adoption and again after hibernation. Subordinate queens may stay in the nest as hopeful reproductives, leave the nest alone or together with workers from the natal nest to start a new colony, or are expelled through attacks by dominant queens and/or the workers (Ito 1990, 2005, Heinze & Ortius 1991, Felke & Buschinger 1999, Trettin et al. 2011). Low-skew populations are abundant in the extended coniferous forests of boreal and alpine Central and Northern Eurasia with a high density of available nest sites for solitary foundresses (Buschinger 1968, Bourke 1991, Stille et al. 1991, Heinze et al. 1995b, Hammond et al. 2006). In contrast, high skew populations with social and reproductive rank orders live in quickly saturated habitat patches with nest site limitation (Ito 1990, 2005, Heinze & Ortius 1991, Felke & Buschinger 1999, Gill et al. 2009, Trettin et al. 2011). The distributional pattern of social organization parallels predictions from optimal skew models in that high skew appears to be associated with ecological constraints on solitary founding (Bourke & Heinze 1994).

We aimed to elicit the formation of rank orders and high reproductive skew in colonies from a low-skew population by deteriorating the environmental conditions for queens. To do so, we experimentally increased queen-worker ratios in natural colonies and/or limited the availability of food. These treatments were motivated by studies according to which queen-worker ratios are considerably higher in functionally monogynous than polygynous species of *Leptothorax* (Heinze 1992) and food shortage may lead to increased skew in both ants (Sommeijer & Van Veen 1990) and social spiders (Salomon et al. 2008). Assuming that queen behavior and reproductive skew are plastic traits, we expected both manipulations to provoke queen-queen aggression and to lead to more pronounced differences in ovarian development. We show that in particular the reduction of worker number leads to queen fighting and more strongly skewed ovarian development.

Materials and Methods

Ant Collection and Rearing

Colonies of *L. acervorum* were collected from their nests in rotting branches in the well-studied low-skew population in Nürnberger Reichswald (June and August 2011), a pine-dominated forest near Nuremberg, Southern Germany (49°16'N, 11°10'E). Whole colonies were extracted from their nests in rotting twigs and transferred into standard three-chambered plastic boxes (10 cm x 10 cm x 3 cm) with plaster floor and reared under artificial spring / autumn conditions (12 h/12 h 20°C/10°C) in incubators as previously described (Heinze & Ortius 1991, Buschinger 1974a).

Experimental Set-up

From freshly collected colonies we set up experimental colonies with 40 workers, 30 brood items and three to a maximum of seven dealate (wingless) queens found in the respective field

colony. Queens were marked individually with 30 µm thin wires (copper or red enameled) tied between alitrunk and petiole, petiole and postpetiole, or postpetiole and gaster. Within three days after the set up, we subjected the experimental colonies to the following four treatments (nine colonies per treatment): control colonies without stressor, food-stressed colonies, severely worker-reduced colonies from which 20 workers were removed at the start of the experiment, and colonies which received a combined treatment of food- and worker-reduction. Colonies were assigned to the four treatment groups so that the averages and the distributions of initial queen numbers did not differ significantly among treatment groups (total initial queen numbers per treatment, control = 44, food-stressed = 49, worker-reduced = 45, food- and worker-reduced = 45; Kruskal-Wallis test, $H_3 = 1.331$, $P = 0.727$; Kolmogorov-Smirnov two-sample tests, all $D < 0.444$, all $P > 0.336$).

Control and worker-reduced colonies were fed three-times during the observation period with chopped cockroaches and diluted honey ad libitum, food-stressed colonies only once. In the field, colonies often experience long periods during which workers cannot forage because of bad weather. Our food limitation experiment did not provide a similarly drastic reduction and colonies can well survive under this condition, albeit without investing a lot of resources into new brood. An observation time of 10 days was chosen as a trade-off, to keep the stressful period as short as possible and, at the same time, to guarantee the minimal sample sizes required to yield statistically meaningful results.

To keep queen-worker ratios high, we removed dark pupae and callow workers from the worker-reduced colonies (with and without food stress) on the fifth day of the observation period. This additional manipulation did not affect the behavior of queens and the frequency of aggression was similar on day five and six in the worker-reduced treatments (Wilcoxon signed rank test, W: $V = 3$, $n_1 = n_2 = 8$, $P = 0.3711$; FW: $V = 3$, $n_1 = n_2 = 9$, $P = 1.0$).

The experiment was carried out during two different observation periods (first round: five colonies per treatment, 2011-07-03 to 2011-07-12; second round: four colonies per treatment,

2011-08-29 to 2011-09-18. During this latter period not all experimental colonies could be simultaneously subjected to the various treatments).

Observation and Ovary Dissection

Observations were started two days after the experimental manipulation. Each colony was observed under a binocular microscope in 20-min sessions twice per day over a period of ten consecutive days (total observation time per colony 400 min). Behavior was recorded by scan sampling every five minutes and in addition by ad libitum sampling (Martin & Bateson 2007). The occurrence of all interactions involving queens (antennal boxing, mandible opening, biting, pulling, stinging/smearing, egg eating, egg laying, grooming, and trophallaxis, i.e., exchange of liquid food) was counted.

After the experiment, we killed all queens by freezing at -20°C and dissected their ovaries under a binocular microscope as described in (Buschinger & Alloway 1978). We noted the presence of sperm in the spermatheca, corpora lutea, and mature oocytes and classified ovarian status as follows (Heinze et al. 1992): I undeveloped ovarioles, II slightly elongated ovarioles with a few immature oocytes, III elongated ovarioles, but no eggs in development (degenerated), IV fully elongated ovarioles with maturing oocytes, V fully elongated ovarioles with corpora lutea, but no eggs in development (degenerated). In addition, we recognized two intermediate stages (II-IV and IV-V).

Eighty-six percent of the observed queens (n = 155) were inseminated, the others were uninseminated and had shed their wings in the field without mating. Behavior of these virgin queens was excluded from the analysis because in *Leptothorax* they take over worker roles (Buschinger 1974b) and consequently do not represent an adequate substitute for mated queens. All colonies used in the analysis contained at least two mated queens except one worker-reduced colony, which was excluded from statistical analysis (for details on queen numbers per colony and treatment see Table S2.2). The exclusion of virgin queens or queens

that died during the experiment led to slightly lowered average queen numbers relative to the beginning of the experiment, but as before neither average queen numbers nor the distribution of queen numbers were significantly different among the four treatments (total final queen number per treatment, control = 38, food-stressed = 45, worker-reduced = 34, food- and worker-reduced = 38; KW-test: $H_3 = 3.1086$, $P = 0.375$; Kolmogorov-Smirnov two sample tests, all $D < 0.333$, all $P > 0.699$).

As was the aim of our manipulation, queen-worker ratios were significantly lower in control/food-stressed colonies than in both types of worker-reduced colonies (Mann-Whitney *U* test: $U = 33.5$, $n_1 = 18$, $n_2 = 17$, $P < 0.001$). Experimental queen-worker ratios in control and food-stressed colonies were well within the range of queen-worker ratios from natural colonies in the low-skew population, while queen-worker ratios in worker-reduced colonies were similar to those previously reported from high skew populations (see Figure S2.1 and Table S2.1; but see Gill et al. 2009). The occurrence of queen mortality and the fact that the reproductive status of queens could only be determined after the observations ultimately resulted in a marginal overlap in queen-worker ratios among treatments (see boxplots A – D in Figure S2.1).

Data Analysis

Preliminary analyses in a subset of colonies showed that egg-laying rate, the frequency of grooming between queens and the rate of aggression and trophallaxis from workers towards queens were too rare to give meaningful results in statistical test. We therefore omitted these types of behavior from the final statistical analysis.

We analyzed the effect of the different treatments on the respective behavioral responses per colony by Scheirer-Ray-Hare tests (SRH), a non-parametric equivalent for a multi-way ANOVA (Sokal & Rohlf 1995), with worker reduction and food reduction as independent factors. To account for the two different observation periods we included “time” as a third

factor. *P*-values were adjusted for multiple tests (queen-queen aggression, egg eating, trophallaxis, grooming) using sequential Bonferroni corrections (Holm 1979). In addition, we used Kruskal-Wallis tests to analyze data for an overall effect of different treatments on queen-queen aggression per colony for each observation period separately. To determine whether queens contribute equally to aggression we estimated the B-index (Nonacs 2000). For the statistical comparison of ovarian development among treatments we combined manipulated colonies due to the small sample size in each category.

All statistical analyses were carried out in R version 2.14.1 to 3.0.1 (R Development Core Team 2011) or PAST v. 1.75b (Hammer et al. 2001).

Ethics statement

As no protected species was sampled and colonies were collected only from a state-owned forest, no permits and approval for ant collection were required. All experiments comply with national and international law.

Results

Queen-Queen and Worker-Queen Behavior

Experimental manipulation of colonies from the low-skew Reichswald population resulted in queen-queen antagonism similar in quality and quantity to that previously observed in high-skew populations (antennal boxing, mandible opening, biting, pulling and stinging/smearing; Heinze & Ortius 1991, Ito 2005, Trettin et al. 2011). In total, we observed 187 attacks among queens during 230 hours of direct observation (107 instances of antennal boxing, 21 threats with opened mandibles, 53 bites, 4 dragging on legs or antennae, 2 stings). The occurrence of aggressive behavior differed greatly among the four treatments and also between the observation periods (Figure 2.1). A Scheirer-Ray-Hare test gave evidence for a strong influence of the factors “observation period” (SRH: $H_1 = 12.68$, $n = 26$, $P = 0.0005$; corrected

for multiple tests, P' = 0.0021) and "worker reduction" (SRH: H_1 = 10.17, P = 0.0014, P' = 0.0057). Averaged over all treatments, more aggression occurred during the second observation period (median, quartiles, first observation period: 0, 0, 0.617 attacks per queen, n = 20; second observation period: 2.167, 0.548, 2.928 attacks per queen, n = 15, Mann-Whitney U test: U = 50, P < 0.0008). Statistical analysis also revealed significant differences between treatments for each observation period separately (Kruskal-Wallis tests, first observation period: H_3 = 10.88, P = 0.0124; second observation period: H_3 = 8.79, P = 0.0323).

In accordance with previous observations (Heinze et al. 1995a), queen-queen aggression almost never occurred in control colonies (median, quartiles 0, 0, 0.2 attacks per queen). The reduction of worker number alone and in combination with food reduction resulted in a considerable increase of aggressive behavior among queens (median, quartiles 2.1, 0.6, 2.9 attacks per queen). In principle, the drastically changed queen-worker ratio, the lower worker number and the experimental removal of workers and worker pupae might all have elicited queen-queen antagonism. However, the following observation suggests that queen aggression is a consequence of manipulated queen-worker ratio and not of disturbance or lower worker number alone: in one of the nine worker-reduced colonies, in which dissection at the end of the experiment revealed that several queens were not inseminated and queen-worker ratio thus was not greatly changed, no aggression was observed. Furthermore, over all worker-reduced colonies, the number of attacks appeared to increase with queen-worker ratio, albeit not significantly so (Gamma correlation: Γ = 0.353, n = 17, P = 0.083). Neither food-reduction nor worker-reduction had a significant effect on the level of aggression from workers towards queens (SRH, food-reduction: H_1 = 1.30, P = 0.255; worker-reduction: H_1 = 0.13, P = 0.717).

Food reduction alone did not have a significant effect on queen aggressiveness (SRH: H_1 = 1.15, P = 0.283, P' = 0.850). In none of the behaviors were interactions among the various factors significant (all P > 0.07). The short duration of the experimental manipulation did not

allow deducing rank orders from the aggression. Nevertheless, the contribution of individual queens to the aggression was significantly different from random in three of those eight colonies with worker reduction (and worker reduction plus food reduction), in which more than 10 aggressive interactions among queens were observed (B-indices of 0.304, 0.978, and 0.214, with the confidence intervals not overlapping 0). Worker reduction in addition led to an increase of egg eating by queens (SRH: $H_1 = 6.19$, $n = 26$, $P = 0.0129$, $P' = 0.0350$, Figure S2.2), and food reduction significantly increased the frequency of trophallaxis between queens (SRH: $H_1 = 9.31$, $n= 27$, $P = 0.0023$, $P' = 0.0091$, Figure S2.3). Queens from worker-reduced colonies were more frequently groomed by workers than queens from food-reduction colonies (SRH: $H_1 = 6.36$, $n = 26$, $P = 0.0117$, $P' = 0.0350$, Figure S2.4).

Ovarian status

Ovarian status differed greatly between the observation periods. The ovaries of queens observed in September were elongated and contained corpora lutea, suggesting that the queens had been fully fertile, but they rarely contained maturing oocytes. Only six of 80 queens had still fully developed ovaries (stage IV), while the majority of queens had ovaries of stage V and probably had started to prepare for hibernation. We therefore did not examine differences in ovarian status among the different treatments in these colonies. In contrast, in July all five control colonies and nine of 14 stressed colonies (3 of 5 colonies with food reduction, 4 of 5 colonies with worker reduction and 2 of 4 colonies with both manipulations), contained one or several fully fertile queens with stage IV ovaries. Four of five control colonies but only two of 14 stressed colonies contained two or more fully fertile queens (Fisher's exact test, $P = 0.017$). Other queens had degenerated their ovaries.

Discussion

We investigated whether queens from a low-skew population of the ant *Leptothorax acervorum* adjust their behavior towards nestmate queens and the partitioning of reproduction in response to experimentally changed conditions. Our data show that experimental manipulation, in particular the reduction of worker numbers, provoked fighting and dominance interactions similar in quality and quantity to those previously observed in high-skew populations of this and other *Leptothorax* species. Though the absolute number of queen-queen attacks was low, few and infrequent interactions may suffice to establish clear social and reproductive rank orders among ants (e. g., Trettin et al. 2011). Food reduction alone did not lead to an increase in aggression but resulted in a higher frequency of food begging and food exchange among queens.

In high skew populations of *L. acervorum* and other functionally monogynous ants queen-queen aggression establishes social rank orders among queens (Heinze & Smith 1990, Heinze et al. 1992, Heinze & Ortius 1991, Ito 2005, Trettin et al. 2011). The incidence of aggression peaks directly after hibernation when queens begin to mature eggs and again in fall when queens prepare for hibernation and young, adopted queens integrate themselves into the hierarchy (Ortius & Heinze 1999). This temporal fluctuation of aggressiveness might explain the large difference in the frequency of queen antagonism between the two observation periods also in our study. The rank order of queens in functionally monogynous ants determines their reproductive status, and usually only the top-ranking queen lays eggs.

Dissections of queens in our experiment indicated that already after ten days of manipulation, queens from worker- and food-stressed colonies differed more strongly in their ovarian status than queens from control colonies. In most stressed colonies, only one queen had remained fully fertile, while other queens had begun to degenerate their ovaries. We cannot exclude that our experimental manipulation directly affected ovarian status in some

queens more than in others. However, comparison with natural high-skew colonies suggests that the observed variation results from aggression and / or an increased rate of food exchange from subordinate to dominant queens. Begging food from subordinates is a subtle mechanism of domination through which queens in related species deplete workers of the resources needed to produce eggs (Franks & Scovell 1983), and food stress was reported to increase reproductive skew in the ant *Myrmica rubra* (Sommeijer & Van Veen 1990). In our study, worker reduction also led to an increased frequency of egg-eating by queens. Egg eating by *L. acervorum* queens is well documented, and queens in low skew populations apparently do not discriminate between their own eggs and those laid by other queens (Bourke 1991, 1994). Such indiscriminate oophagy does not alter stable skew. In contrast, Ito (2005) observed that dominant queens from a high-skew population on Hokkaido fed on eggs immediately after these were laid by subordinate queens and in this way increased reproductive skew. Unfortunately, we did not determine the origin of eggs in our study, but it is possible that egg eating would have increased skew in stressed colonies.

Ideally we should also have performed the opposite experiment, i.e., trying to induce queen tolerance in colonies from high skew populations of *L. acervorum* by adding workers or overfeeding the colonies. However, documenting the complete disappearance of a rare behavioral trait is more difficult than documenting its induction. Furthermore, queen aggressiveness and reproductive skew appear to vary to some extent among natural colonies of high skew populations of *L. acervorum* and related species, and not in all studied colonies were queens seen to engage in aggressive interactions (Gill et al. 2009, Heinze & Ortius 1991, Trettin et al. 2011, Gill & Hammond 2011).

Our result that an increase of the queen-worker ratio increases queen-queen antagonism and reproductive skew matches the observation that queen-worker ratio is higher in high-skew species than in low-skew species (Heinze 1992). Queen-worker ratio might co-vary with ecological constraints on solitary colony founding that have been suggested to affect the

magnitude of reproductive skew. A high queen-worker ratio can result from a high re-adoption rate of young queens or high mortality of workers, e. g., during hibernation. Both might reflect adverse environmental conditions, which pose high costs on dispersal and colony founding by solitary queens (Bourke & Heinze 1994). In addition, the chance for individual queens to found new colonies accompanied by workers might be more limited at higher queen-worker ratios.

The easy induction of queen-queen aggression and increased reproductive skew in colonies in which queens normally do not fight is at odds with results from previous studies on social plasticity in ants. For example, the social structure of colonies of the red imported fire ant, *Solenopsis invicta*, depends on the genotypes of queens and workers at a particular locus (Gotzek & Ross 2007), and whether co-founding queens of *Messor pergandei* and *Pogonomyrmex californicus* exhibited aggression or were mutually tolerant did not depend on group size but on region of origin (Helms et al. 2013, Overson et al. 2013). Similarly, queens of *Temnothorax rugatulus* were not capable of expanding their behavior to that shown during the workerless colony founding phase under experimental worker shortage (Rüppell et al. 2002). Our finding also contrasts with previous studies in *L. acervorum*: dissections and population genetic analyses suggest that the level of skew is more or less fixed within populations, and that colonies maintain their social phenotype when kept under standardized laboratory conditions (Heinze et al. 1995a & b, Gill et al. 2009, Hammond et al. 2006). Therefore, different levels of reproductive skew have been suggested to be an evolved rather than a behavioral response (Gill et al. 2009, Gill & Hammond 2011). This discrepancy might mean that ants from different populations have evolved different thresholds for fighting and dominance behavior, leading to more or less fixed local social organization. In addition, variation in skew within populations may have been underestimated by genetic analyses and dissections. Skew estimates from the genetic assignment of maternity of adults are often difficult because of the typically high relatedness among nestmate queens and rapid queen

turnover. Genotyping of eggs and dissections give only a snap-shot account of the status quo of the partitioning of brood production. As it may be difficult to distinguish between formerly active, but now degenerate ovaries and not yet activated ovaries, the past or future contributions of queens that presently do not mature eggs may be underrated.

Conclusions

Our study demonstrates that queens of *L. acervorum* from low skew populations are able to react to changes in their social environment. In addition, it highlights the importance of queen-worker ratio for the adjustment of skew and the need for further studies to clarify its role and the role of other factors (e. g., egg cannibalism and habitat structure) in the formation and maintenance of reproductive skew in insect societies.

Acknowledgements

We thank Lisa Marie Braun and Martin Bircheneder for assistance during experimental preparation and observation.

Author Contributions

Conceived and designed the experiments: JH, JT. Performed the experiments: TS, JT. Analyzed the data: JT, TS. Wrote the paper: JT, JH.

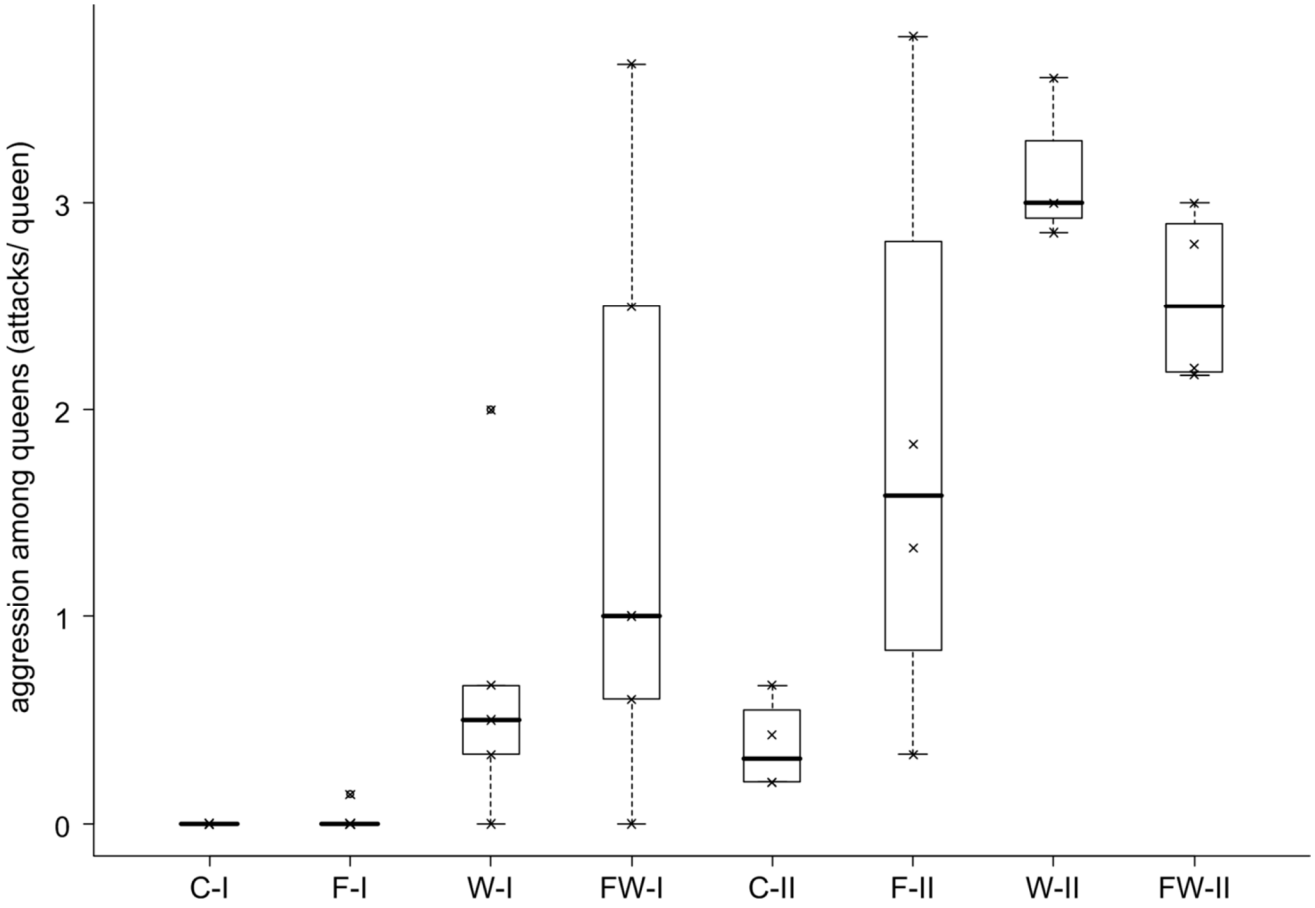

Figure 2.1 Frequency of aggression among queens of the ant *Leptothorax acervorum* from a low-skew population. Data shown as attacks per queen during the total observation period (median, quartiles, range). Individual colonies were subjected to different types of stress (food reduction F, worker reduction W, or both FW) or left unmanipulated (control C) in two different seasons (July, I, and September, II). Outliers are indicated as ○ and original data points as ×.

CHAPTER 3

Phylogeography of a socially plastic ant in SW-Europe

Jürgen Trettin* and Jürgen Heinze

Biology 1, University of Regensburg, Regensburg, Germany

Abstract

In this study, we investigated ultimate factors underlying variation in reproductive skew in the ant *Leptothorax acervorum* (Hymenoptera: Formicidae) within a phylogeographic framework. Colonies of this socially polymorphic species contain multiple breeding queens over large parts of its Holarctic range, while in peripheral populations usually only one queen per colony reproduces. In particular, we address the question of whether variation in social organization is associated with a strong barrier to gene flow and thus indicating reproductive isolation between both social forms of *L. acervorum* in SW-Europe. In addition, we conducted an extensive analysis of the specie's evolutionary history, phylogeography and demography in the Iberian Peninsula and adjacent areas. Our analyses revealed no strong association of genetic differentiation and divergence with social organization of *L. acervorum*, indicating a lack of reproductive isolation between both social forms. In contrast, our results suggest that demographic and spatial patterns in genetic variation and differentiation are better explained by co-variation with climatic, ecological and historical factors.

Introduction

The Quaternary climate changes and their dramatic effects on demography and distributional ranges left behind their footprints on the evolution of Palearctic biota. During the Ice Ages, polar ice sheets and the surrounding permafrost extended southward and caused major changes in species' distributions (Bennett 1997), either by range shifts or local and regional extinctions (cf. Habel et al. 2010). While boreal species could survive in the periglacial areas even during the last glacial maximum (LGM 23-18 ka BP), temperate species retreated mainly to suitable habitats further south, in particular the Mediterranean peninsulas of Iberia, Italy and Balkans (Hewitt 2004). Recolonization routes of Northern and Mid Europe took by boreal and temperate species after the LGM led to the typical patterns in the distribution of species and intraspecific genetic variation found today (e. g., contact and hybrid zones in Mid Europe due to expansion and secondary contact of populations from different southern refugia, cf. Schmitt 2007 and Hewitt 2011a). In addition, some populations could survive in mountainous regions of Central and Southern Europe (e. g., Alps, Carpathians, Caucasus or Pyrenees) by ascending with the climate and their habitat, leading to the establishment of mountain endemic and arctic-alpine or boreal-montane disjunct species (Schmitt 2009, Schmitt & Varga 2012). Relict populations of boreal species thus “trapped” in their Mediterranean refugia may be exposed to different environmental conditions than populations from the center of the range and can be (partially) isolated from the latter (Cassel-Lundhagen 2010). This can lead to the accumulation of unique genotypes and special adaptations in peripheral populations (e. g., Eckert et al. 2008, Heino & Hanski 2001, cf. Cassel-Lundhagen 2010 and references therein).

The Holarctic ant *Leptothorax acervorum* (Hymenoptera, Formicidae) has mainly a boreo-montane distribution in Europe, and ranges from the northernmost tip of continental Scandinavia to southern European mountains. Its highest colony densities are found in light

coniferous forests (mainly *Pinus sylvestris*), where it nests under bark or in decaying branches close to the ground (Seifert 2007, Czechowski 2012). *L. acervorum* reaches the south-western most edge of its Palearctic distribution in mountainous pinewoods of northern and central Iberia at elevations above 1500m a. s. l. (Felke & Buschinger 1999, Gill et al. 2009, Trettin 2011).

Interestingly, peripheral populations of *L. acervorum* differ from central populations in colony structure. Across its whole range, colonies can contain one or more queens, but the partitioning of reproduction among nestmate queens ("reproductive skew") differs strikingly (Heinze et al. 1995b, Gill et al. 2009). In colonies from most of boreal and temperate Eurasia all nestmate queens reproduce (facultative polygyny), whereas queens in colonies from the range margin (e. g. Japan or Central Spain) form social and reproductive hierarchies and only the top-ranking queen lays eggs (functional monogyny, Ito 1990, Felke & Buschinger 1999, Gill et al. 2009, Trettin 2011). In addition, mating behavior differs between populations from Central Spain and Central Europe (cf. Felke & Buschinger 1999 for a detailed discussion).

The aims of this study were (i) to map the distribution of both social forms of *L. acervorum* in inner Iberian mountains and Pyrenees, (ii) to analyze the microevolutionary history of both social forms using genetic markers and, finally, to infer the population history of *L. acervorum* in SW-Europe in a broader phylogeographic context.

Materials & Methods

Ant sampling and DNA extraction

Colonies of *Leptothorax acervorum* were collected between 2008 and 2011 in the Iberian Peninsula (SG, SA, SD, SNW I and II), the Pyrenees (PY I and II), southern France (FR I to V), southern England (E) and southern Germany (D) (see Table 3.1 & Figure 3.1 for details). In total, 105 colonies (one worker each) from the Iberian Peninsula and the Pyrenees were genotyped for ten polymorphic nuclear microsatellite loci. The mitochondrial DNA dataset

contains sequences of 62 workers from all sampled locations and geographic regions (Table 3.1). All specimens were preserved in 95% ethanol or frozen at -20°C until extraction. Genomic DNA was isolated from individual workers using a cetylmethylammoniumbromide (CTAB)-protocol (Sambrook and Russell 2001).

Ant keeping and ovary dissection

For the determination of social structure and reproductive skew, we dissected a total of 528 queens from 66 colonies from Pyrenees (PY) and four Iberian populations (SNW I, SNW II, SG, SD). Ovary dissections were conducted right after transfer of colonies to the laboratory (August 2009: SG; November 2009: PY, SD; February 2010: SD; July 2010: SNW I & II, SD). All queens were killed by freezing at -20°C and ovaries were subsequently dissected under a binocular microscope as described before (Buschinger & Alloway 1978). We noted the presence of sperm in the spermatheca, corpora lutea, and mature oocytes and classified ovarian status as described in Trettin et al. (2014) and Heinze et al. (1992).

Reproductive skew in the different populations was specified by recording the number of colonies with no queen, one queen (monogynous), more than one fertile queen (polygynous) or only one fertile queen among several in inseminated, but non-laying female reproductives (functionally monogynous, sensu Buschinger 1968). For completeness we added literature data from a fifth Iberian population (SA, Felke &Buschinger 1999, Gill et al. 2009, Trettin et al. 2011)

Microsatellite analysis

Four of the ten used microsatellite loci had been developed specifically for *L. acervorum* (GA1, GA2, GT1 & GT2; Bourke et al. 1997), the remaining loci represent cross-amplifications from different ant species (GT218 & GT223; Hamaguchi & Ito 1993, 2MS67; Suefuji et al. 2011, 2MS46; Suefuji et al. unpublished – 2MS46fwd: 5'-

GCTCACTACTATGCTGCCAGC -3', 2MS46rev: 5'- TCCGTCTATCCCTTCCTGCAA -3', L18; Foitzik et al. 1997 and Myrt3a; Evans 1993). PCR conditions were chosen as follows: initial denaturation 5 min at 95 °C; 35 cycles of 60 s at 95 °C, 45 s at the locus-specific annealing temperature of 45-60 °C, elongation of 45 s at 72 °C and a locus specific final extension step of 30-120 s at 72 °C. Total reaction volume was 20 µL, of which 1 µL was DNA template. PCR products were either analyzed on an ABI PRISM 310 automated sequencer (GA1, GA2, GT218 & GT223) and subsequently genotyped using GENESCAN 3.1 (Applied Biosystems) or sent for analyses to GATC Biotech AG (GT1, GT2, L18, Myrt3a, 2MS67 & 2MS46 (II)) and subsequently genotyped using PEAK SCANNER v1.0 (Applied Biosystems).

Each microsatellite locus was tested for deviation from Hardy-Weinberg equilibrium (HWE) using exact HW tests (Weir 1996). For testing of independence between loci we assessed linkage disequilibrium (LD) for all locus pairs across populations (Fisher's method). Both tests were run in GENEPOP 4.2.2 (Rousset 2008). Evidence for the occurrence of null alleles was assessed and corrected using the program MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004). We found evidence for null alleles in 14 of out of 70 microsatellite loci x population pairs, of which only three pairs had null allele frequencies larger than 0.2 (maximum: 0.243, see Table S3.1 for details).

Original and null allele corrected datasets were both used in downstream analyses of population substructure (i.e., F-Statistics and neighbor- joining trees). Analysis of both datasets resulted only in marginal differences in global and pair-wise F_{ST} – values and micro-satellite-tree topologies (null allele corrected global F_{ST} = 0.074 and see Figure 3.2A, Table 3.6 and Appendix: Figure S3.1 and Table S3.2). We, therefore, decided to perform all down-stream analyses with the original microsatellite dataset if not explicitly stated otherwise.

Population genetic differentiation and structure (microsatellites)

Number of alleles (*k*), allelic richness (*A*), number of private alleles (A_P) as well as expected (H_E) and observed (H_O) heterozygosities were calculated per population and locus using FSTAT 2.9.3.2 (Goudet 1995) and GENALEX 6.5 (Peakall & Smouse 2012). The genealogical relationships among study populations were analyzed by reconstructing neighbor-joining trees in POPULATIONS 1.2.31 (Langella 1999) using D_A distance (Nei et al. 1983). Bootstrap values were obtained by 2000 replications over loci. To study population structure in more detail we estimated overall and pairwise F-statistics using GENEPOP 4.2.2 (Rousset 2008) as well as GENALEX 6.5 (Peakall & Smouse 2012) to calculate allelic-diversity corrected F_{ST}-value analogs (Hedrick's standardized G_{ST}, G_{ST} corrected for small sample sizes G''_{ST}, and Jost's *D*, Jost 2008). To test the hypothesis that genetic differentiation within regions is equal or higher than the genetic differentiation between regions (regions defined here as geographic regions – Iberian Peninsula (IB) and Pyrenees (PY) or high skew vs. low skew populations) we conducted an analysis of molecular variance (AMOVA) using ARLEQUIN v3.5.1.2 (Excoffier &Lischer 2010). Significance of results was evaluated over 10000 replicates.

We applied Bayesian clustering to assess fine-scale population genetic structure (as well as identifying distinct populations) – an approach capable of assigning individuals to populations without a priori knowledge, based upon multi-locus genotypic data. Cluster analysis was run in STRUCTURE 2.3.4 (Prichard et al. 2000) allowing individuals to have mixed ancestry (admixture model) without using sampling locations as prior information and without correlated allele frequencies among populations. As recommended by Prichard et al. 2000 (cf. Structure documentation), we run a first analysis including microsatellite data from all locations and in a second analysis excluded samples from the most divergent population (PY II). Potential population cluster values (*K*) varied from 1 to 10 with ten runs per value of *K*, burn-in and sampling period were set to 300,000 (first analysis) and to 200,000 generations

(second analysis) accordingly. For each analysis the optimal *K* value was assessed by following the ΔK-method as described by Evanno et al. (2005) and implemented in the program STRUCTURE HARVESTER web-v0.6.93 (Dent & van Holdt 2012).The program DISTRUCT v1.1 (Rosenberg 2004) was used to graphically display the output.

Finally, we used a Mantel test (Mantel 1967) to evaluate genetic isolation by distance in the microsatellite data, with significance of results evaluated by 999 matrix permutations in GENALEX 6.5 (Peakall & Smouse 2012). Genetic distance matrix among populations was calculated as linearized F_{ST}-values [(F_{ST} / (1- F_{ST})] in FSTAT 2.9.3.2 (Goudet 1995) whereas geographical distances among populations in kilometers were calculated in GENALEX 6.5.

Demographic history

Because of the patchy distribution of *L. acervorum* in "mountainous islands" in central Spain, we tested for the occurrence of bottlenecks in each sampled population. First, we used Wilcoxon's sign rank test, which tests for an excess of heterozygosity, implemented in BOTTLENECK 1.2.02 (Piry et al. 1999). The test was performed assuming the strict stepwise mutation model (SMM) and the two-phase model (TPM) with probability of single-step mutations set to 95%. The variance for mutation size was set to 12 in both cases as recommended by Piry et al. (1999) and Peery et al. (2012) respectively. Altogether 110,000 simulations were run.

We used the *M*-ratio statistic (Garza & Williamson 2001) to test for a severe reduction in effective population size. The *M*-ratio is calculated from the number of alleles and the allelic range per locus whereby the number of alleles is expected to decline faster than the allelic range during a bottleneck. The mean *M*-ratio per population was calculated in ARLEQUIN v3.5.1.2 (Excoffier &Lischer 2010). Ninety-five percent confidence intervals (CI) of the *M*-ratio were obtained by bootstrapping over loci for 9,999 replicates using the *boot* package in *R* (R Development Core Team 2011). We compared observed *M*-ratios to the upper limits of

empirically derived *M*-values after a bottleneck (= 0.68; Garza & Williamson (2001) and to critical *M* values (M_c) simulated from populations in mutation-drift equilibrium. We used the following parameter settings: eight values of θ ranging from 0.02 to 200 [where $\theta = 4N_e\mu$; N_e is the pre-bottleneck effective population size and μ the microsatellite mutation rate, herein assumed to be in the order of 10^{-4} per locus and generation (see Bhargava &Fuentes 2010 for a review)], the mean size of larger mutations; δ_g = 3.5 and the proportion of multi-step mutations; p_g = 0.2. Both parameters specify how similar the chosen microsatellite mutation model (i. e., herein a 20% TPM) is to a strict single-step SMM. All simulations were run with 10,000 replicates per population and θ in the program CRITICAL M (Garza 2011).

Mitochondrial DNA analyses

The primers C1-J- 2183 and A8-N-3914 (Simon et al. 1994) were used to amplify an approximately 1641 bp long mitochondrial DNA fragment, starting from within the COI gene, including the complete COII sequence, and finishing in the very beginning of the ATPase 8 gene. PCR was carried out in a total reaction volume of 15 µL using the BIO-X-ACT Short Mix (Bioline) and 1 µL DNA template. PCR conditions consisted of an initial denaturation 4 min at 94 °C; 38 cycles of 75 s at 94 °C, 75 s at 50 °C (annealing), elongation of 150 s at 72 °C, and a final extension step of 5 min at 72 °C. PCR products were sent to LGC Genomics for purification and Sanger sequencing.

The chromatograms were assessed and edited using CHROMAS LITE 2.1.1 (Technelysium) and subsequently concatenated in BIOEDIT (Hall 1999). Sequences were aligned manually and automatically by using the algorithm CLUSTAL W as implemented in BIOEDIT (Larkin & Blackshields 2007). All sequences could be aligned unambiguously, and no indels were found except in three sequences from the eastern Pyrenees (PY I) that contained a 1bp-deletion. Since this appeared in a noncoding region only, it was considered valid and was used as a fifth mutational state in network analysis. The absence of unusual stop codons as potential

evidence for the presence of pseudogenes (numts, e. g., Song et al. 2008) was checked in the ARTEMIS GENOME BROWSER using the invertebrate mitochondrial codon table (Rutherford et al. 2000). Thereafter, the aligned sequences were used for further phylogeographic and population genetic analysis.

Phylogeography and population structure

To evaluate the genealogical relationships among sampled populations we reconstructed haplotype networks using the statistical parsimony algorithm as implemented in TCS (Clement et al. 2000). The reconstructed network contains two extreme divergent haplotypes, including the three sequences with a 1bp-deletion as well as a highly divergent sequence from FR III. These haplotypes are similarly divergent as a reference sequence (same primer pair and PCR conditions used) from the closely related socially parasitic species *Leptothorax kutteri* (Figure 3.3). Due to their unclear taxonomic status these sequences, together with all non-French and -Iberian sequences, were removed from all downstream analysis.

For the quantification of genetic polymorphism we used the following standard diversity indices: number of haplotypes (h), number of private haplotypes (h_P), number of segregating sites (S), haplotype diversity (H) and nucleotide diversity (π) for each locality, for each geographic region, and for the whole COI data set except the extremely divergent haplotypes excluded) using DNASP v5.10.01 (Librado & Rozas 2009). We estimated the amount of genetic differentiation for the whole dataset and among geographic regions, following Pons & Petit (1996) by calculating Φ_{ST} – values from mean pairwise differences (N_{ST}) and haplotype frequencies (G_{ST}) in ARLEQUIN v3.5.1.2 (Excoffier & Lischer 2010). By comparing both statistics of genetic differentiation it is possible to test whether the mtDNA-sequence dataset contains a signal of phylogeographic structure (if $N_{ST} >> G_{ST}$) beyond that in haplotype frequencies alone (following the rational of Pons & Petit 1996, for a review see Garrick et al 2010). An additional AMOVA was carried out to test the hypothesis that genetic differentia-

tion within regions (Φ_{SC}) is equal or higher than the genetic differentiation between regions (Φ_{CT}, the definition of regions follows as described for the microsatellite data). In case both measures are equal, their ratio is expected to be one. In contrast, if their ratio is larger than one, $\Phi_{SC} > \Phi_{CT}$. Significance of AMOVA results was evaluated over 10000 replicates.

mtDNA demographic history

Tests for selective neutrality (Tajima's *D* and Fu's F_S) were performed for each region and for the whole dataset (after excluding samples from Germany, England and the extremely divergent haplotypes) to measure whether data deviate from expectations under a model of neutral evolution or equilibrium population size, respectively. Significance of neutrality tests was evaluated over 10,000 replicates in ARLEQUIN v3.5.1.2 (Excoffier &Lischer 2010). In addition, we evaluated the demographic history of *L. acervorum* from SW-Europe by plotting the distribution of pairwise differences between haplotypes (mismatch distribution, Rogers & Harpending 1992) for each region and for the combined dataset. Afterwards, we compared the fit of observed mismatch distributions to simulated distributions from a sudden expansion model. All calculations were done in ARLEQUIN v3.5.1.2 (Excoffier &Lischer 2010), using 1000 replicates to evaluate the significance of results.

Results

Ovary dissections and reproductive skew

Of the 528 female reproductives from 66 *L. acervorum* colonies from four Iberian populations and the Pyrenees 400 were inseminated and 120 were virgin female sexuals that nevertheless had shed their wings. Spermathecal content could not be determined in eight individuals. Eighty percent of the Iberian colonies (43 out of 54, Table 3.2) were functionally monogynous and contained only one functional queen plus several mated, but non-laying queens. The remaining 11 colonies from Iberia were either monogynous or queenless (five vs.

six colonies). In contrast, half of the Pyrenean colonies (six out of twelve, Table 3.2) had multiple reproductive queens (polygyny) and half were monogynous or queenless (two vs. four). Functional monogyny of the dissected Iberian colonies is in agreement with previously published data for the Iberian population SA and SG (Table 3.2; Buschinger & Felke 1999, Gill et al. 2009, Trettin et al. 2011).

Genetic diversity indices

Significant deviation from HWE after Bonferroni correction for multiple tests (α = 0.00071, Appendix: Table S3.3) was found in only two of 70 microsatellite locus x population combinations. No evidence of linkage disequilibrium was found for any combination of loci. Thus, we decided to retain all samples and loci in downstream analysis.

No clear spatial pattern was observed for H_O or H_E (Table 3.3). In contrast, the Iberian populations seem to have lower values of k (range: 6.3 to 9.5), A_P (range: 3 to 14) and A_R (range: 6.0 to 9.0) than the Pyrenean populations (MWU-tests for k, A_P and A_R each: U = 10, N_1 = 2, N_2 = 5, $P < 0.1$).

Genetic diversity at the mtDNA fragment did not differ strongly between geographic regions (Table 3.4). Haplotype diversity in the total dataset was generally high ($H_{D:}$ 0.934) and ranged from 0 to 1.0 among populations.

Population structure and phylogeography

Analysis of genealogical relationships among populations resulted in an unrooted neighbor-joining tree with nodes supported by bootstrap values between 10 and 96% (Figure 3.2A), which reflects the geographic distribution of populations within major Iberian mountain systems: Pyrenees (PY I & II), Cantabrian Mountains (SNW I & II) and Iberian System (SA, SG, SD). The longest internal branch separates the two Pyrenean populations

from the remaining Iberian populations. The population from the western Pyrenees (PY II) diverges most among all analyzed populations.

Total genetic differentiation (F_{ST}) was low (0.075) and remained modest even after correction for multiple alleles (G'_{ST}: 0.257, G''_{ST}: 0.265, D_{est}: 0.205; all values significant with $P < 0.01$). Again the population from the western Pyrenees showed a higher differentiation than all inner Iberian populations (Table 3.5). Genetic differentiation within regions was approximately three times higher than genetic differentiation between regions (Figure 3.4).

STRUCTURE runs using all locations strongly supported $K = 2$ genetic clusters (ΔK_2: 250.6, ΔK_3: 8.6; Figure S3.2A). With a probability of ≥80%, 92% of the individuals from the inner Iberian populations could be assigned to the first cluster, and 95 % of the individuals from the western Pyrenees (PY II) could be assigned to the second cluster (Figure 3.2B). In contrast, genotypes from the eastern Pyrenees (PY I) suggested pronounced admixture (Figure 3.2B). A cluster analysis without the western Pyrenean samples supported four genetic clusters ($K = 4$) within the remaining microsatellite dataset (ΔK_4: 5.9, next smaller ΔK_2: 5.0, for details see Figure S3.2B). Overall, the level of admixture was higher among the remaining populations (Figure 3.2C). In particular, eastern Pyrenees (PY I), eastern Cantabria (SNW I) and Sierra de Gúdar (SG) showed evidence of high admixture. However, 70% of the individuals from the remaining Iberian System (SA and SD) could be assigned to a third cluster with a probability ≥80%, while 67% of the samples from western Cantabria (SNW II) could be assigned to a fourth cluster. Tests for associations between genetic and geographic distances (isolation by distance) in the microsatellite data were non-significant for Euclidean ($r_{xy} = 0.010$, $P > 0.1$) and log-transformed Euclidean distance ($r_{xy} = 0.083$, $P > 0.1$).

The reconstructed mtDNA-haplotype network has a decentralized structure and shows a short to intermediate expanded genealogy with only reduced geographic segregation of haplotypes (i. e., phylogeographic structure, Figure 3.3). The only exception are two

extremely divergent haplotypes from the eastern Pyrenees (PY I) and a nearby population from France (FR III), which differ by 31 and 26 mutations, respectively, from the core network. In total, the decentralized network comprises 28 haplotypes (22 haplotypes without extremely divergent, English, and German haplotypes), including three dominant haplotypes: the first is predominantly distributed in southern France and western Pyrenees (7+1 sequences, among them two sequences from FR III), the second from the Pyrenees (6+1 sequences from PY I and PY II respectively) and the third predominant in inner Iberia (five sequences in total). The remaining French samples forming a second predominant French haplogroup (Figure 3.3).

Significant genetic differentiation (Φ_{ST} estimated as G_{ST} and N_{ST}) was observed for the whole dataset (IB-PY-FR, extremely divergent haplotypes excluded) and for all pairwise combinations of geographic regions (IB, PY and FR; Table 3.6, all with $P < 0.01$). The global analysis without regional partitioning of data revealed moderate genetic structure within the whole dataset as well ($\Phi_{ST} = 0.498$, $P < 0.001$). The data did not reveal a strong phylogeographic structure (i. e., N_{ST} is never larger than G_{ST}) in pairwise comparisons of regions or the whole dataset (Table 3.6 and Figure 3.3 for similar results on network structure). In addition, genetic differentiation within regions was higher than genetic differentiation among regions for all combinations of regions, with $\Phi_{SC} : \Phi_{CT}$ ratios > 2 (range: 2.17 – 9.22, Figure 3.4).

Demographic history

Analyses of recent demographic events by summary statistical tests of microsatellite variation provide evidence for demographic bottlenecks. Results from the Wilcoxon test in BOTTLENECK showed that two inner Iberian populations have a significant excess of heterozygosity only, indicating a recent bottleneck (95% TPM, SG: $P = 0.0015$; SA: $P = 0.0093$). In contrast, *M*-ratio tests suggest that all populations have experienced bottlenecks, with observed values well below most critical values for all but the most extreme values of θ

(i. e. θ > 20, corresponding to an effective pre-bottleneck population size of some 50000 individuals, Figure 3.5), including the threshold of the empirically derived *M*-value of 0.68.

Analyses of historical demographic events using summary statistical tests of mtDNA variation provided no significant evidence for demographic changes or selective events within each geographic region (Table 3.4). In addition, mismatch distributions for each region are multimodal and values of raggedness are at least by the factor of 2.6 larger (even if non-significant) than the value for the total dataset (raggedness; FR: 0.067, PY: 0.098 and IB: 0.120; for mismatch distributions see Figure S3.3). In contrast, analyses of demographic history for the total dataset suggest a sudden population expansion (mismatch distribution: unimodal, nonsignificant raggedness coefficient: 0.026 and a significant negative value of Fu's F_S but no significant value of Tajima's *D*, Table 3.4).

Discussion

Spatial distribution: behavioral polymorphism and genetic structure

In this study we provide data on the distribution of the two social forms of the ant *Leptothorax acervorum* in the Iberian Peninsula and Pyrenees. We show that colonies are functionally monogynous over all studied inner Iberian mountain ranges, as previously shown for the populations of Sierra de Albarracin (SA) and Sierra de Gúdar (SG) (Felke and Buschinger 1999; Gill & Hammond 2011, Trettin et al. 2011). In contrast, colonies from the Pyrenees are facultatively polygynous.

On a first glance, our population genetics analysis seems to support an association of genetic differentiation and social organization in *L. acervorum*. However, more detailed population genetics analysis shows stronger genetic differentiation within than between social forms (Figure 3.4 & Table 3.6) for both nuclear and mitochondrial markers. In particular, western Pyrenees (PY II) revealed the highest pairwise differentiation to all inner Iberian populations while individuals from eastern Pyrenees (PY I) revealed pronounced admixture

from both genetic clusters. Furthermore, haplotype network analysis (mtDNA) failed to find a clear pattern of differentiation between the two social forms. No significant IBD pattern between microsatellite genetic differentiation and geographic distance was found.

Overall, these results suggest a lack of reproductive isolation between both social forms in the western Palearctic range of *L. acervorum*. Observations on colonies of both social forms (Gill et al. 2009) have previously suggested genetic differences underlying the variation in social structure, while the genetic analysis could not substantiate a differentiation between both phenotypes at neutral loci. In particular, the authors highlighted the fact that both social forms maintained their respective phenotype when kept under common laboratory conditions. In contrast, behavioral experiments with facultatively polygynous colonies recently have shown that queen antagonism can be elicited by manipulation of queen's social-environment and hence suggest that queen behavior and social phenotype are plastic and adjustable in response to environmental changes (Trettin et al. 2014).

It remains unknown whether the behavior of queens from the functionally monogynous populations from the marginal range of the species is similarly flexible or whether they have lost their behavioral plasticity by canalization (Schmalhausen 1986; originally published in English in 1949), and see West-Eberhard 2003 and references therein) or an evolutionarily changed threshold for the elicitation of queen fighting behavior. Furthermore, a lack of genome resolution (10 microsatellite loci + one mtDNA locus) might be responsible for the observed lack of genetic differentiation between both social forms. For instance, queen number in the red imported fire ant *Solenopsis invicta* is under control of non-recombining "social chromosomes", with limited differentiation between them (Wang et al. 2013). Nevertheless, wherever a genetic basis for social polymorphism has been suggested (i. e., via genetic differentiation, phenotypic stability or genomic rearrangements) there is also evidence for significant mtDNA differentiation between the different phenotypes (for a literature review see Table S3.4), suggesting that a pattern of mtDNA differentiation between both

social forms is an reliable indicator for a genetic basis underlying social polymorphism. Nevertheless, in addition to our phylogeographic study cross-fostering experiments, breeding pedigrees, genome scans or functional genomic analyses are needed to determine definitively to which extent social organization in *L. acervorum* is under genetic control or represents environmentally induced plasticity.

Phylogeography of L. acervorum in Iberian Peninsula

The high biodiversity of the Iberian Peninsula and hence its role as southern European refugium for the postglacial recolonization of Europe has long been recognized in phylogeography (e. g., Hewitt 1996, 2001, 2011b, Taberlet et al. 1998). In addition, recent studies on inner Iberian phylogeography revealed a substantial geographic complexity in genetic diversity, divergence and extent of admixture among and within many species, leading to the establishment of the "refugia within refugia" concept (see Gomez & Lunt 2007, Hewitt 2011b and Nieto Feliner 2011 for a review). The difficulties in deriving general conclusions from these complex patterns seem to be the result of a minimization of dramatic effects caused by climatic oscillations (e. g., minimized extinction of genotypes, populations and species; Nieto Feliner 2011).

In particular, the fragmented Iberian landscape with several mountain ranges and river basins allowed the survival of populations in multiple refugia and simultaneously their partial differentiation in allopatry, followed by secondary contact between previously differentiated genomes (Nieto Feliner 2011). In contrast to climate induced range shifts in Central and Northern Europe the recurrent cycles of "reproductive isolation and secondary contact" happened in the Iberian Peninsula across smaller scales, primarily by altitudinal range shifts (Hewitt 2011b, Nieto Feliner 2011). Whether these inner Iberian glacial refugia were mainly composed of completely isolated populations (Gomez & Lunt 2007) or may be better

understood primarily as a patchwork of temporarily interconnected populations across glacial-interglacial cycles (Nieto Feliner 2011) is still subject of an ongoing discussion.

Patterns of genetic diversity and genetic structure in *L. acervorum* in inner Iberian mountains can be associated with landscape features as well as climatic and demographic history. On a local scale, genetic differentiation in microsatellites is associated with Iberian mountains, suggesting no current gene flow between and reduced gene flow within main mountain systems. In addition, the patchy distributions of private alleles (A_P) and populations showing high vs. low admixture (PY I, SG & SNW I vs. PY II, SNW II, SA & SD) within Iberian mountains are in accordance with the existence of multiple refugia within and varying connectivity among them throughout the late Pleistocene. In contrast, analysis of mtDNA data did not reveal a pronounced genetic structure between Iberian populations and mountain systems respectively.

The most western Cantabrian population (SNW II) provides a notable exception to this pattern. It is characterized by a distinct mtDNA haplotype and a distinct genetic cluster for the multi-locus microsatellite data, suggesting a longer period of evolution in isolation. This resembles the situation in the capercaillie (*Tetrao urogallus*) a bird characteristic of Eurasian coniferous forests (Duriez et al. 2007, Rodríguez-Muñoz et al. 2007, Storch 2007). In contrast, its Cantabrian population inhabits deciduous forests and is the most genetically distinct and depauperate in Europe except for the Pyrenean population, to which it is closely related (Rubiales et al. 2008, Blanco-Fontao et al. 2010).

In the Iberian Peninsula, *L. acervorum* is found almost exclusively in mountainous pinewoods or pine-dominated forests above 1500m a. s. l., suggesting a close association of the species with the history of highland pines, in particular *Pinus sylvestris*. We suggest that *L. acervorum* populations co-expanded and -contracted with *P. sylvestris*-dominated forests during glacial and interglacial states respectively, suggesting larger population sizes and higher levels of gene flow among inner Iberian populations during glacial and transitional

phases (e. g., early Holocene). In contrast, the predominant biogeographic process acting during the last inter-glacial would be fragmentation and decline of populations (i. e. micro-vicariance) in concert with Iberian lowland pinewoods (e. g., in central Ebro basin). Consequently, these populations within inner Iberian mountains may represent relict populations of this cold-adapted ant species (Seifert 2007, Czechowski et al. 2012), within an inter-glacial southern European refugia.

Relationships of Iberian L. acervorum to the adjacent regions (Pyrenees, W-Alps and central Europe)

In contrast to the clear signal of population structure (microsatellites) found among Iberian populations, mtDNA sequence data contains no signal of phylogeographic structure and shows similar levels of genetic diversity between geographic regions. These results are in accordance with former phylogeographic studies on *L. acervorum*, where low phylogeographic structure between populations from large parts of Europe was found too (Brandt et al. 2007, Foitzik et al. 2009, Gill et al.2009, but see Foitzik et al. 2009 for a contrasting result on microsatellite genetic structure in *L. acervorum*).

In addition, the data does not support the existence of a single SW-European refugium contributing alone to the postglacial recolonization of Central and Western Europe. In contrast, the data imply the existence of two glacial refugia in France alone: the (i) extended at least from the lower Rhône valley westward to the foothills and lowlands north of the Pyrenees and (ii) in the upper Rhône valley or adjacent areas north of it (e. g., around the Jura and Vosges mountains). Evidence for the existence of glacial refugia north of the Mediterranean peninsulas has been recently recognized for several plant and animal species (Stewart & Lister 2001, Birks & Willis 2008, Provan & Bennett 2008, Schmitt & Varga 2012, but see Tzedakis et al. 2013 for a different perspective on northern tree refugia). In particular artic and alpine species, widespread in the periglacial areas but with current northern European

and/or fragmented distributions between European high mountain systems, show patterns of genetic association similar to *L. acervorum* in southern and central France (e. g., Schmitt 2009, Schmitt & Varga 2012). In southern France, for example, populations of taxa from French Massif Central (MC) often show a phylogeographic association with neighboring high mountains (i. e., SW-Alps and the Pyrenees) indicating postglacial recolonization of MC either from at least one glacial refugium in the lowlands between them or from at least one refugium north of MC (Schmitt 2009). Several other studies have found evidence of refugia in southern France as well (e. g., see Kropf et al. 2012 for a review), including ants (Leppänen et al. 2011). In addition, evidence for the existence of Extra-Mediterranean refugia in central Europe (e. g. central France, Vosges or Black Forest) is accumulating for many cold-adapted species (Schmitt & Seitz 2001, Fink et al. 2004, Ursenbacher et al. 2006, Huck et al. 2009, Drees et al. 2010, Hammouti et al. 2010, Leppänen et al. 2013 among others).

Subsequent to the late glacial (post-10 ka BP) the first French refugium may have started to fragment and *L. acervorum* shifted its range towards more suitable habitats in the surrounding mountains and their foothills, while at the same time populations of the second refugium would have expanded their range at least towards MC and western Alps. This scenario is supported by one haplotype associated with the first refugium, found exclusively in the western Pyrenees (PY II), southernmost Massif Central (FR III) and Mont Ventoux (FR IV), while the second refugium is indicated by a haplogroup found on the remaining French localities (FR I, II & V). In addition, a closely related haplotype from England suggests a direct connection to the first French refugium (including two sequences differing by one mutation only from the main haplotype of the latter, Figure 3.3) e. g., via the contribution of populations from the first refugium to the postglacial recolonization of NW-Europe.

Analogous scenarios of postglacial range dynamics were described for many species from the high mountain ranges of Europe (see Schmitt 2009 for a review). In case of the mountain butterfly *Erebia epiphron*, patterns of genetic structure within and between W-European high

mountains suggest (i) recolonization of the central and western Pyrenees and western Alps from refugial lowlands in between and (ii) of the east Pyrenees from the unglaciated foothills south-east and east of it (Schmitt et al. 2006).

Similar to the latter scenario, our analysis revealed a differential contribution of the first French refugium and the inner Iberian refugial system to the postglacial recolonization of Pyrenees not just in colonization routes but also in patterns of gene flow by both genetic markers. In contrast to the French refugium, inner Iberian populations share no haplotypes with the Pyrenees (in particular the eastern Pyrenees) while microsatellite data indicates historical gene flow between inner Iberia and eastern Pyrenees, but not between western Pyrenees and inner Iberian populations. These patterns suggest that during the early Holocene *L. acervorum* still could maintain gene flow between unglaciated parts of eastern Pyrenees (PY I) and mountains of the Iberian System (e.g. via a network of suitable habitat patches in the Ebro basin), while subsequently gene flow gradually broke down in parallel with the increasing aridification and the following continual decline of pinewoods in the Ebro basin during the second half of Holocene (e. g. Davis & Stevenson 2007, Carrión et al. 2010, Rubiales et al. 2010, 2012, Pérez-Obiol et al. 2011).

In addition, no or only limited gene flow occurred between inner Iberia and western Pyrenees (PY II) at the same time, a pattern explainable by the stronger influence of oceanic climate and consequently the replacement of pinewoods by broad-leafed tree taxa in the Cantabrian Mountains and western Pyrenees (Crawford 2005, Carrión et al. 2010, Rubiales et al. 2012). Finally, the lack of congruence in both molecular markers might suggest male-biased gene flow and limited female dispersal between inner Iberian and eastern Pyrenean populations, a pattern commonly found in ants (Sanetra & Crozier 2003, Sundström et al. 2005, Barth et al. 2013, Soare et al. 2014). This conclusion is further supported by *L. acervorum*'s mating behavior, for which the species shows a similar plasticity as in the case of number of queens per colony (see Felke & Buschinger 1999, for detailed discussion).

L. acervorum's demographic history in SW-Europe

Because of *L. acervorum's* fragmented distribution in Iberian mountains, we tested for the occurrence of bottlenecks among the populations of that region. Slightly lower values of microsatellite genetic diversity of inner Iberian compared to Pyrenean populations (i. e. *k* vs. H_E & H_O, Table 3.3) indicated already potential bottlenecks in the former populations (Cornuet & Luikart 1996). In contrast, our analysis found very low *M*-ratios for all populations, including the Pyrenees, indicating that they went through sever bottlenecks in the recent past. All observed *M*-ratios were far below the critical *M* value of 0.68 (as derived by Garza & Williamson 2001) and when compared to simulated critical *M* values, all seven populations seem to have experienced bottlenecks at values of θ (i.e. mutation rate scaled effective population size) that are reasonable large for *L. acervorum* (θ = 20–40, correspond to pre-bottleneck N_e of ~50,000-100,000). In addition, heterozygosity excess test confirmed the occurrence of severe bottlenecks in two populations from the Iberian system (SA and SG).

The different results of both tests for the remaining five populations can be explained by differences in test procedures and the low power of the heterozygosity excess test due to low sample size or deviations in mutation models (Piry et al. 1999, Peery et al. 2012).

In contrast, mtDNA demographic analyses (summary statistical tests, mismatch distributions, raggedness) provide no clear evidence for any kind of historical change in effective population sizes or selective events.

Overall, the occurrence of bottlenecks in the recent past is in accordance with the late and postglacial range contractions or shifts of *L. acervorum* in the Iberian peninsula, as indicated by both genetic datasets (for detailed discussion, see above). Finally our results highlight the vulnerability of *L. acvervorum* in Iberian mid Mountain ranges to climate change and human induced climatic warming in particular, where the species inhabits suitable microhabitat patches over 1500m a. s. l. only.

Conclusion

In the present study, we found no strong association of genetic differentiation and divergence between both social forms of *L. acervorum* in SW-Europe, indicating a lack of reproductive isolation between low and high skew populations. In contrast, our results suggest that patterns of population structure and haplotype distribution are better explained by co-variation with climatic, ecological and historical factors, such as quaternary climate changes, oceanity-continental gradient, heterogeneous topography of Iberian Peninsula, microclimate conditions and other local effects. Moreover, these patterns suggest that reduction in gene flow between currently in mountains isolated populations of *L. acervorum* is a rather recent phenomenon, except for the populations form the most western Cantabrian Mountains. The distribution of mtDNA haplotypes indicates that *L. acervorum* from French refugia took part on the postglacial recolonization of Central and Western Europe, in contrast to inner Iberian populations. Finally, our study reveals the occurrence of recent bottlenecks in inner Iberian and Pyrenean populations and, thus, highlights the vulnerability of *L. acervorum* in SW-European Mountains to climate change and human induced climatic warming.

Acknowledgements

We thank I. Rakitchenkova, M. Schneider, I. Miranda da Silva and A. Trindl for helping during laboratory work; M. Suefuji for kindly providing unpublished microsatellite primers; A. Buschinger, M. Füßl, M. Haubner, S. Hutschenreuther, P. Koller, R. Landstorfer, S. Leopold, D. Rothgänger, G. Schneider, I. Miranda da Silva, T. Seyferth and C. Wanke for assistance during field sampling, and S. Agrawal, A. Keikhosravi, C. Löser, C. Oberprieler, C. Schubart and N. Thiercelin for helpful discussions and suggestions. The work was financed by Deutsche Forschungsgemeinschaft (He 1623/25).

Author Contributions

Conceived and designed the study: JH, JT. Performed the field work: JT, JH. Analyzed the data: JT. Wrote the paper: JT, JH.

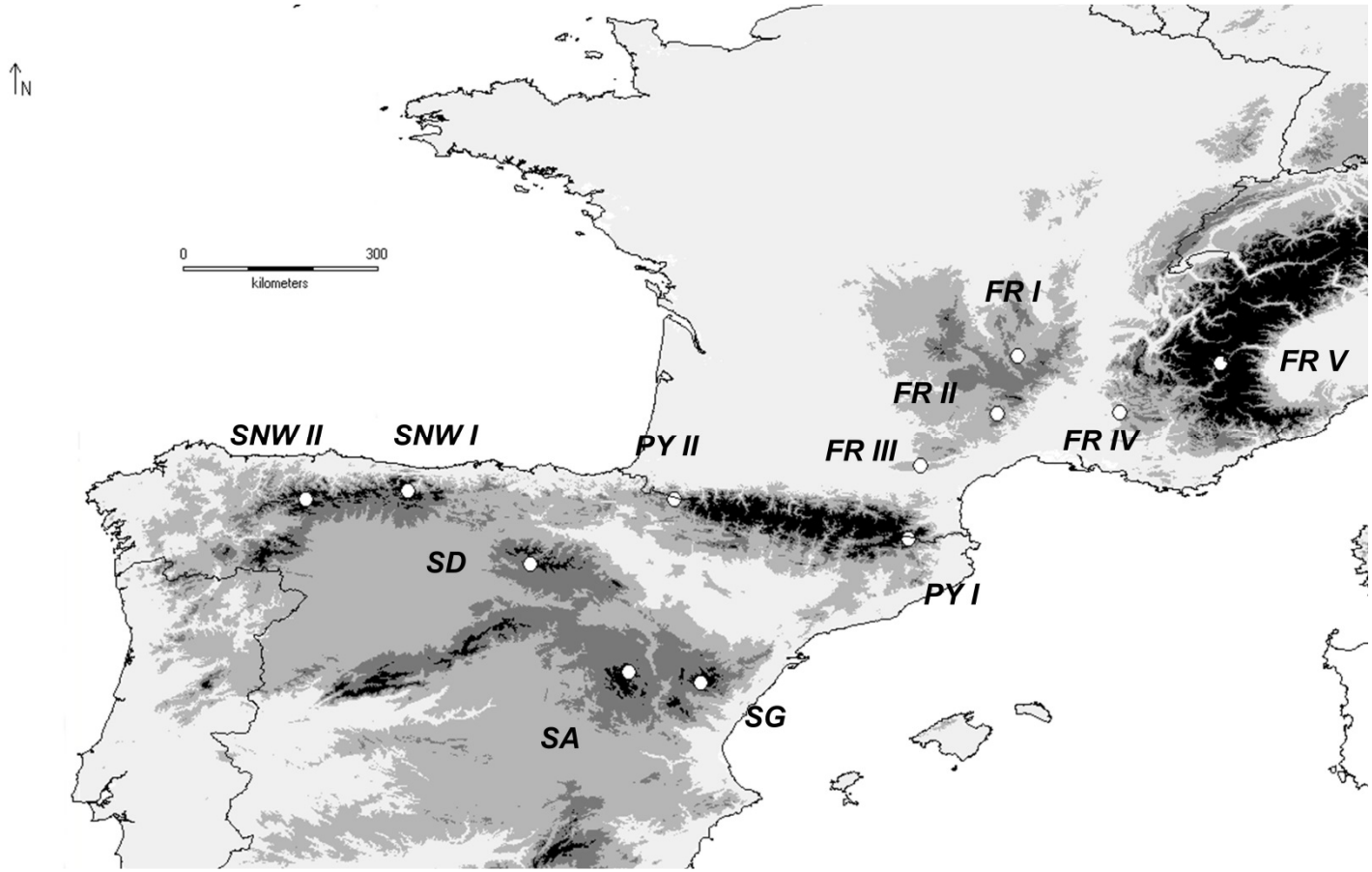

Figure 3.1 Map showing sampling locations of *L. acervorum* in SW-Europe (white circles). For details on sample ID see Table 3.1. Elevation levels (m a. s. l.) are given in different shades (black: above 1500, dark grey: 1000 – 1500, grey: 500 – 1000, light grey: 0 – 500).

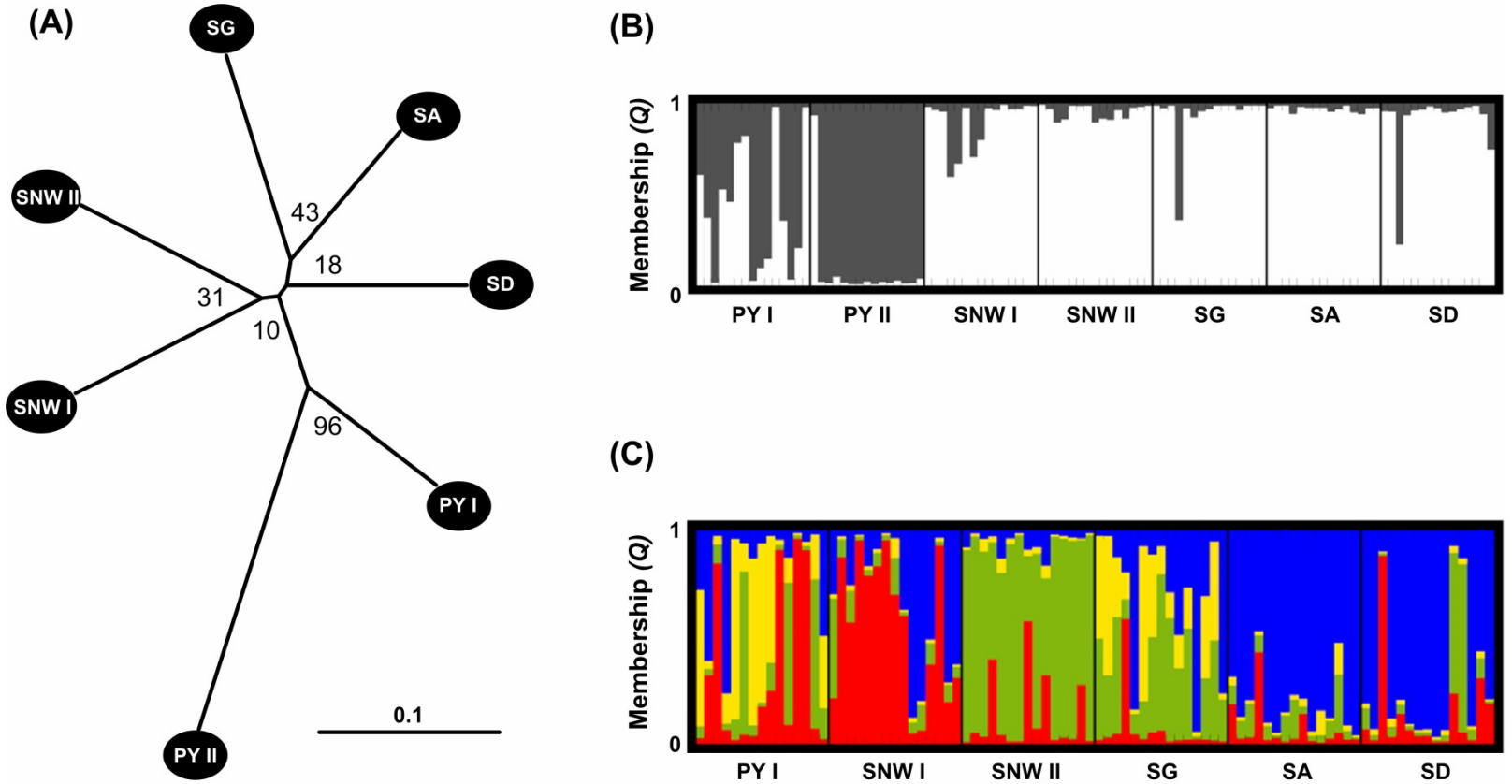

Figure 3.2 Analysis of genetic structure and relationship between and within populations (for microsatellites). (A) Unrooted neighbor-joining tree using Nei's D_A distance, with bootstrap values given as numbers close to nodes. (B) Bayesian assignment analysis with all samples included (individual membership proportions for K = 2 given in different shades). (C) Bayesian assignment analysis without samples from PY II (individual membership proportions for K = 4 given in different colors). For population's ID and location see Table 3.1 and Figure 3.1.

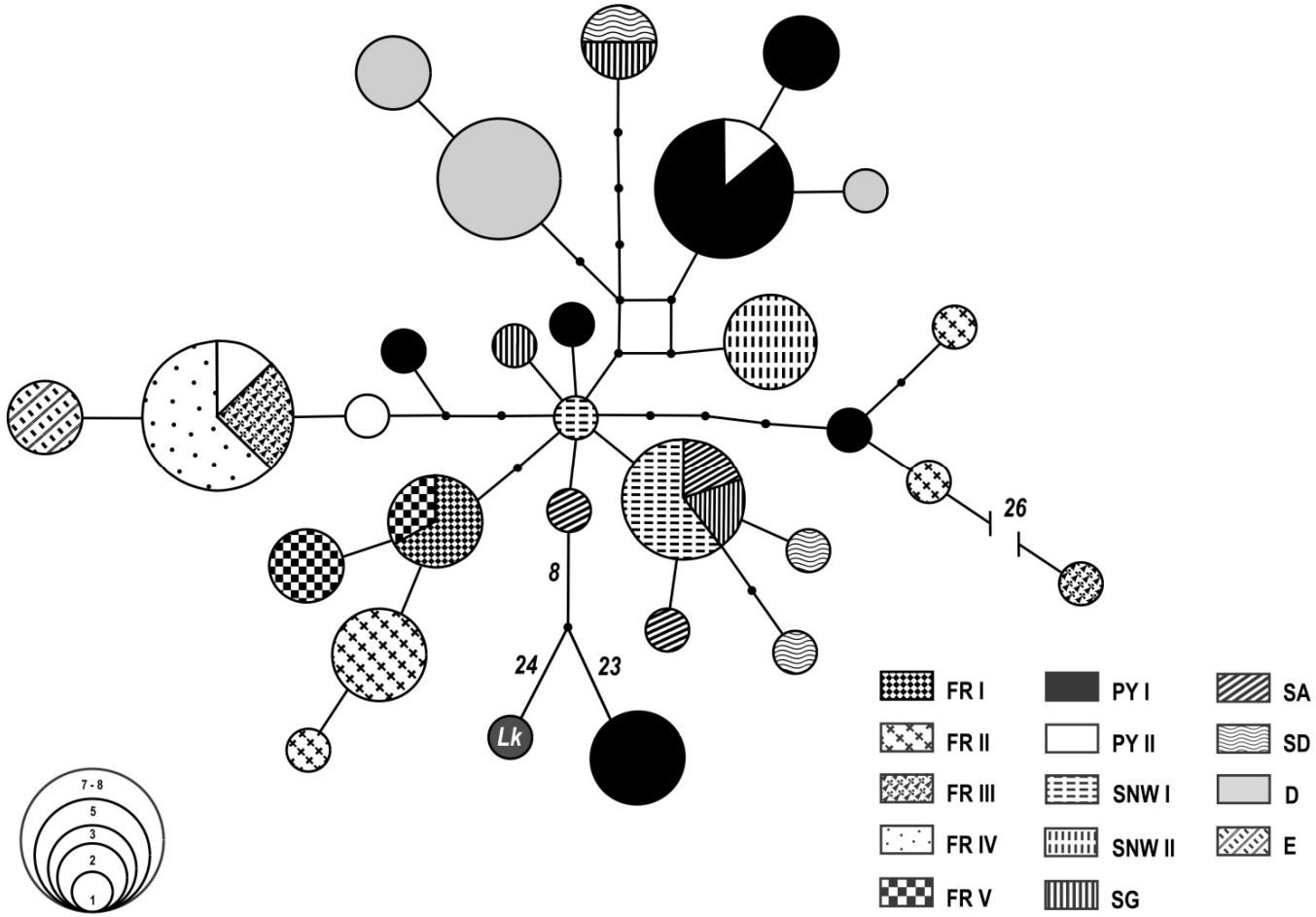

Figure 3.3 Haplotype network of *Leptothorax acervorum* from SW-Europe, Germany (D) and England (E). Locations are labeled by different patterns and shades (for details on locations see Table 3.1 and Figure 3.1). The size of haplotypes is proportional to the number of individuals and each line between haplotypes represents a single mutational step. Included are two extreme divergent haplotypes that differ by 31 (PY I) and 26 (FR III) mutations and a reference sequence of *Leptothorax kutteri* (*Lk*) that differ by 32 mutations from core network, respectively.

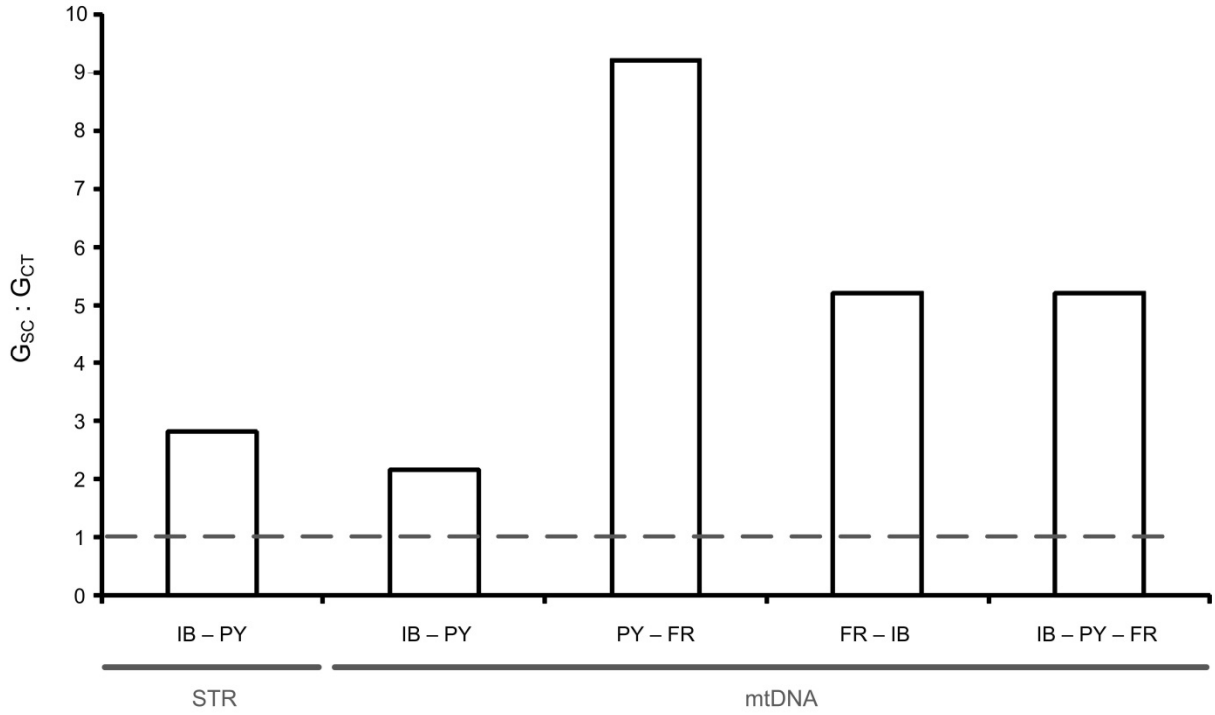

Figure 3.4 Pairwise comparison of genetic differentiation within versus between geographic regions, defined as G_{SC} : G_{CT} ratio and calculated within an AMOVA framework. Dashed line indicates 1:1 ratio, above which differentiation within regions is larger than between regional differentiation. Comparison on microsatellite data (STR) included the IB – PY regional pair only, while comparison on mtDNA data included all regional pairs. Additionally, a comparison including mtDNA sequences from all regions was conducted (IB – PY – FR).

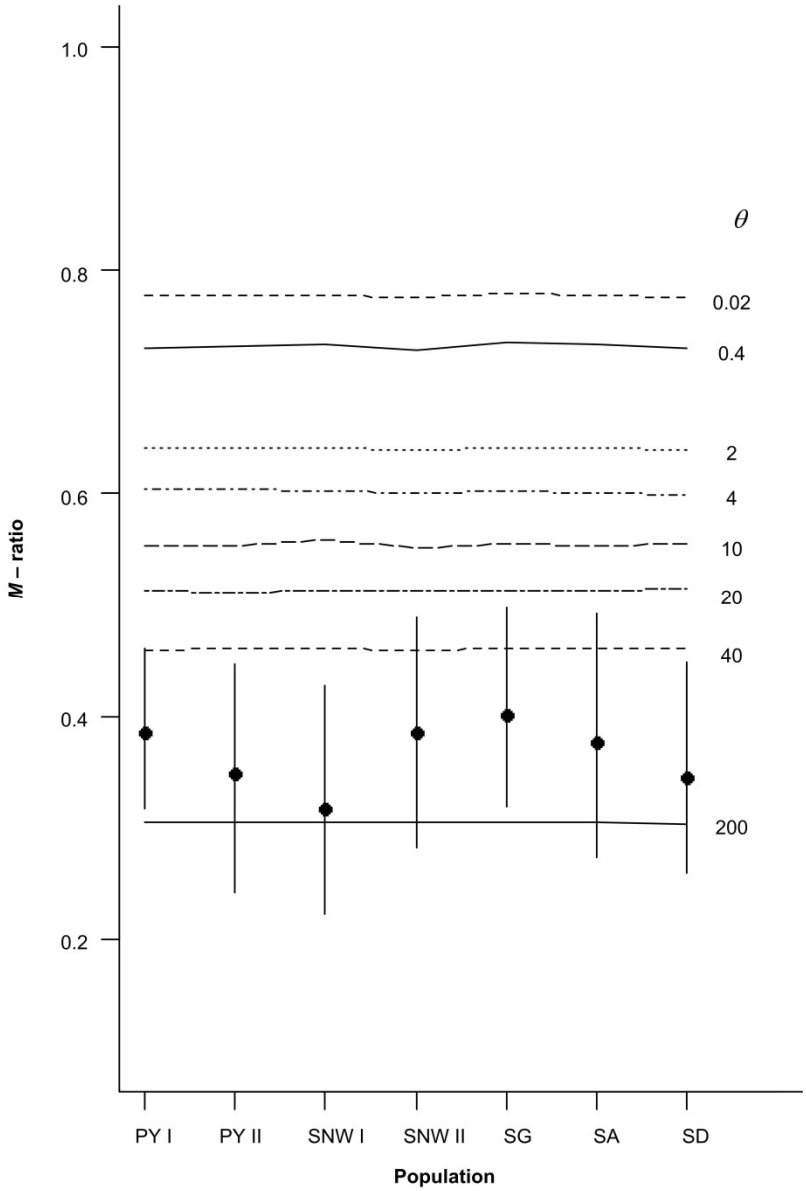

Figure 3.5 Observed *M*-ratio for each study population (closed circles with 95% CI indicated as vertical lines). Horizontal lines indicate critical threshold values of *M* (M_C) simulated for equilibrium populations at different θ-values.

Table 3.1 Sampling information for the study area of *Leptothorax acervorum in* SW-Europe, Germany and England. Population ID, geographic location, collection year, geographic region and number of samples per population.

ID	Region	Location (N, E)	Year	No. of samples (SSR / mtDNA)
PY I	Pyrenees	42.401°, 2.288°	2009	15 / 14
PY II	Pyrenees	42.953°, -1.012°	2009	15 / 3
SNW I	Inner Iberian	43.067°, -4.766°	2010	15 / 4
SNW II	Inner Iberian	42.963°, -6.197°	2010	15 / 3
SG	Inner Iberian	40.371°, -0.627°	2009	15 / 3
SA	Inner Iberian	40.525°, -1.647°	2008, 2009, 2010	15 / 3
SD	Inner Iberian	42.044°, -3.040°	2009, 2010	15 / 3
FR I	southern France	44.985°, 3.840°	2010	* / 2
FR II	southern France	44.176°, 3.541°	2010	* / 6
FR III	southern France	43.433°, 2.471°	2010	* / 3
FR IV	southern France	44.182°, 5.275°	2010	* / 5
FR V	southern France	44.877°, 6.688°	2010	* / 3
D	southern Germany	49.266°, 11.168°	2011	* / 8
E	southern England	§	§	* / 2

* Populations not genotyped for microsatellites. § See Brandt et al. (2007) for details on sequences.

Table 3.2 Reproductive status of queens and reproductive skew of colonies of *L. acervorum* from inner Iberian mountains and Pyrenees.

Population	*n*	Queenless	Monogyne	Facultative polygyne	Functional monogyne	Reproductive skew
PY	12	2	4	6	0	low
SNW I	7	0	2	0	5	high
SNW II	11	3	0	0	8	high
SG	11	2	1	0	8	high
SD (2009)	15	0	2	0	13	high
SD (2010)	10	1	0	0	9	high
SA*	-	-	-	-	-	high

* SA: data from Felke & Buschinger (1999), Gill et al. (2009), Trettin et al. (2011), *n*: number of colonies.

Table 3.3. Genetic diversity indices for 10 microsatellite loci in Iberian populations of *L. acervorum*.

Region	Population	*n*	*k*	A_P	A_R	H_O	H_E
Pyrenees	PY I	15	9.5	11	8.96	0.646	0.803
	PY II	15	8.1	14	7.59	0.665	0.686
Inner Iberian	SNW I	15	7.1	9	6.73	0.571	0.672
	SNW II	15	6.3	4	6.00	0.670	0.687
	SG	15	6.5	3	6.27	0.598	0.741
	SA	15	6.7	8	6.42	0.624	0.722
	SD	15	6.9	4	6.55	0.607	0.710

n: no. of individual workers, *k*: no. of alleles, A_P: no. of private alleles, A_R: allelic richness, H_O: observed heterozygosity, H_E: expected heterozygosity

Table 3.4 Genetic diversity indices and neutrality tests for mtDNA sequences per studied region and location of *L. acervorum* in SW-Europe (*sequences PY07, PY08, PY09, SNW15.b and FR3.2 excluded*).

Region	Population	*n*	*h*	h_P	*H*	π	*S*	Tajima's *D*	Fu's F_S
Pyrenees (PY)		14	7	6	0.758	0.0024	14	-0.360	-0.092
	PY I	11	5	4	0.709	0.0020			
	PY II	3	3	1	1.000	0.0033			
Inner Iberian (IB)		15	8	8	0.867	0.0023	15	-0.761	-1.026
	SNW I	3	1	0	0	0			
	SNW II	3	1	1	0	0			
	SG	3	3	1	1.000	0.0033			
	SA	3	3	2	1.000	0.0012			
	SD	3	3	2	1.000	0.0041			
Southern France (FR)		18	7	6	0.817	0.0030	16	0.227	0.384
	FR I	2	1	0	0	0			
	FR II	6	4	4	0.800	0.0031			
	FR III	2	1	0	0	0			
	FR IV	5	1	0	0	0			
	FR V	3	2	1	0.667	0.0004			
Total	PY+IB+FR	47	21	n.a.	0.934	0.0033	32	-0.889	-6.922*
England	E	2	1	1	0	0			
Germany	D	8	3	3	0.607	0.0010			

n: no. of sequences, *h*: no. of haplotypes, h_P: no. of private haplotypes, *H*: haplotype diversity, π: nucleotide diversity, *S*: no. of segregating sites, * $P < 0.02$

Table 3.5 F_{ST} – values for Pyrenean and inner Iberian population pairs of *L. acervorum*, calculated from 10 microsatellite loci.

ID	PY I	PY II	SNW I	SNW II	SG	SA
PY II	0.065					
SNW I	0.071	0.118				
SNW II	0.060	0.120	0.087			
SG	0.039	0.128	0.086	0.068		
SA	0.049	0.113	0.050	0.078	0.044	
SD	0.055	0.101	0.044	0.090	0.056	0.019

For details on population ID and location see Table 3.1and Figure 3.1.

Table 3.6 Results of analysis of molecular variance (AMOVA) by pairwise comparison between geographic regions for *L. acervorum* calculated from haplotype frequencies (G_{ST}) and pairwise differences (N_{ST}, values in brackets) respectively (with extreme divergent haplotypes excluded). Values for null allele corrected microsatellite data (*STR*) in brackets. For a definition of geographic regions see text.

Regional pairs	Fixation index			Percentage of variation		
STR	F_{CT}	F_{SC}	F_{ST}	V_A	V_B	V_C
IB – PY	0.024 (0.026)	0.068 (0.068)***	0.090 (0.092)***	2.40 (2.58)	6.60 (6.63)	90.99 (90.80)
mtDNA	Φ_{CT}	Φ_{SC}	Φ_{ST}	V_A	V_B	V_C
IB – PY	0.107 (0.162)	0.232** (0.231*)	0.314 (0.355)***	10.65 (16.20)	20.76 (19.33)	68.59 (64.47)
PY – FR	0.041 (0.123)	0.378 (0.506)***	0.403 (0.567)***	4.08 (12.30)	36.23 (44.41)	59.69 (43.29)
FR – IB	0.088* (0.186**)	0.459 (0.506)***	0.507 (0.597)***	8.14 (8.75)	35.12 (41.92)	56.74 (49.33)
IB – PY – FR	0.070 (0.154*)	0.365 (0.430)***	0.410*** (0.251**)	7.01 (15.35)	33.97 (36.40)	59.02 (48.25)

V_A /Φ_{CT}/F_{CT}*:* among regions relative to the total population, V_B /Φ_{SC}/F_{SC}*:* among local populations within regions, V_C /Φ_{ST}/ F_{ST}*:* among local populations relative to the total population, significance levels: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, asterisks outside brackets refer to both values per fixation index.

CHAPTER 4

Genetic or social environmental basis underlying queen behavioral variation – a laboratory transplantation experiment

Jürgen Trettin and Jürgen Heinze

Biologie 1, Institut für Zoologie, Universität Regensburg, D-93040 Regensburg, Germany

Abstract

In this study, we conducted a transplantation experiment to investigate whether queens' social environment may affect their behavior towards other queens or, alternatively, whether factors underlying variation in queens' behavior have a genetic basis only, in low and high skew colonies of the ant species *Leptothorax acervorum*.

Introduction

In animal societies often reproduction is not equally shared among group members (i. e. reproductive skew) and consequently conflict over reproductive participation may arise (cf. Keller & Reeve 1994, Nonacs & Hager 2011, Reeve & Shen 2013). Depending on the particular species or even populations, reproductive skew can be low (if reproduction is equally partitioned among group members) or high (if only one or a few individuals dominate reproduction). Among other factors, variation in reproductive skew can be affected by ecological constraints on solitary nesting, the relatedness and social status of group members, (cf. Keller & Reeve 1994, Johnstone 2000, Vehrencamp 1983, Nonacs & Hager 2011). For example, in high skew species individual's reproductive share is often determined by its social status (i. e. dominant versus subordinate), which in turn may depend on its fighting abilities. Despite its pronounced interspecific variation, reproductive skew usually varies less within species or populations (Jamieson 1997, Kutsukake & Nunn 2006).

In most social insects, queens in mature multi-queen societies show little antagonism and contribute equally to the offspring of the group (‘‘polygyny’’). High or even maximal skew has been described only from a small number of species with multi-queen colonies, where only one of several inseminated queens monopolizes reproduction (Pardi 1946, Buschinger 1968, Heinze & Smith 1990, Yamauchi et al. 2007). This ‘‘functional monogyny’’ (Buschinger 1968) results from the formation of social rank orders among potential reproductives through overtly aggressive or ritualized dominance behavior (e.g. Pardi 1946, Heinze & Smith 1990, Heinze et al. 1992, Reeve 2000, Yamauchi et al. 2007,).

Nonetheless, intraspecific variation in social structure (including reproductive skew) has been described recently from several social insect species (Ross & Keller 1998, Felke & Buschinger 1999, Chapuisat et al. 2004, Bargum et al. 2007, Helms & Helms-Cahan 2012, Soro et al. 2010, Overson et al. 2013). These examples contain transitions from solitary to

social life styles (e.g. in sweat bees, Field et al. 2010) or variation in queen number per colonies of eusocial insects (e .g., Ross & Keller 1998, Felke & Buschinger 1999, Chapuisat et al. 2004). In addition, various factors underlying this variation in reproductive skew were proposed by the authors that can be assigned to three types: (i) a strong genetic basis, (ii) a strong influence of environmental cues and (iii) the influence of species' social environment (e. g. via maternal or sib effects) or a combination of all three factors.

The Holarctic ant *Leptothorax acervorum* is widely distributed over large parts of the northern hemisphere (Francoeur 1983, Seifert 2007, Czechowski 2012). Colonies are facultatively polygynous in the extended coniferous forests of Central Europe and Siberia (Buschinger 1968, Bourke 1991, Heinze et al. 1995a & b), but functionally monogynous where they are only patchily distributed, i.e., on sun exposed slopes in Alaska, in light clearings in Hokkaido, and at the southern limit of its range in mountainous areas in central Spain (Ito 1990, Heinze & Ortius 1991, Felke & Buschinger 1999, Gill et al. 2009).

In this study, we focus on the latter factor underlying variation in reproductive skew by trying to determine whether the social environment of queens may affect their behavior (in particular aggression) towards other queens of the species *L. acervorum* in an laboratory full-factorial transplantation experiment.

Material & Methods

Collection and laboratory maintenance

Colonies of *L. acervorum* were collected from their nests in rotting branches from (i) the low-skew population in Nürnberger Reichswald (during several sampling trips between 2008 and 2011), Southern Germany (49°16'N, 11°10'E) and (ii) the high skew population in S^{ra} de Albarracin (during several sampling trips between 2008 and 2010), a mid-sized mountain range in central Spain (40°30'N, 1°38'W). Whole colonies were extracted from their nests in rotting twigs and transferred into standard three-chambered plastic boxes (10 cm x 10 cm x 3

cm) with plaster floor and reared under artificial winter conditions (12 h/12 h 5°C/0°C) in incubators as previously described by Heinze & Ortius (1991) and Buschinger (1974a).

Experimental Set-up

From low- and high skew stock colonies we set up experimental colonies with 20-30 workers, 10-20 brood items and four dealate (wingless) queens from the respective stock colonies, according a full-factorial design. Queens were marked individually with 30 µm thin wires (copper or red enameled) tied between alitrunk and petiole, petiole and postpetiole, or postpetiole and gaster. Subsequently, we allocated the marked queens to the following eight treatments (eight colonies per treatment): control colonies from Reichswald (CR) and S^{ra} de Albarracin (CS), one queen each from four different Reichswald colonies with workers from yet four other Reichswald colonies (4RR), one queen each from four different S^{ra} de Albarracin colonies with workers from four different Reichswald colonies (4SR), one queen each from two different S^{ra} de Albarracin colonies and from two different Reichswald colonies with workers from four different Reichswald colonies (22R) and the respective combinations of queens with workers from S^{ra} de Albarracin (4SS, 4RS, 22S). To exclude 'foreignness of queens' in treatment colonies as a source of aggression we kept all experimental colonies under winter conditions (12 h/12 h 5°C/0°C) for additional three to eight weeks.

Observation and Ovary Dissection

After that hibernation phase rearing conditions of the first replicate colonies were changed to spring conditions (12 h/12 h 20°C/10°C). Observations started one day after change to spring conditions and each colony was observed under a binocular microscope in 30-min (replicates 1 to 3) respectively 20-min (replicates 4 to 8) sessions twice per day over a period of ten consecutive days (total observation time per colony 600 respectively 400 min). Behavior was recorded by scan sampling every five minutes and in addition by ad libitum

sampling (Martin & Bateson 2007). The occurrence of all interactions involving queens (antennal boxing, mandible opening, biting, pulling, stinging/smearing, egg eating, egg laying, grooming, and trophallaxis, i.e., exchange of liquid food) was counted.

After the observation period, we killed the queens by freezing them at -20°C and dissected their ovaries under a binocular microscope to check for ovarian status. Dissections were carried out as described in Buschinger & Alloway (1978). We noted the presence of maturing oocytes, corpora lutea, and sperm in the spermatheca. Ovarian status was classified following Heinze et al. (1992). The experiment was carried out during two different observation periods (first round: two colonies per treatment, 2010-04-26 to 2010-05-19; second round: six colonies per treatment, 2011-03-26 to 2011-06-03). Ants were fed with honey and cockroaches twice weekly and water ad libitum. The descriptive statistical analyses were carried out in Excel (Microsoft 2010) and *R* version 3.0.1.

Results and Discussion

Forty-six percent of the observed queens were inseminated (n = 118, range = 2 – 24 per replicate), the others were uninseminated and had shed their wings in the field without mating or could not be dissected. Behavior of these virgin queens was excluded from the analysis and colonies used in the analysis contained at least two mated queens.

On a first glance, our results imply that both the origin of queens and the origin of workers play an important role on the level of aggression among queens and hence the determination of skew in *L. acervorum* (Figure 4.1). Queens from high skew populations were less aggressive in a low skew than in a high skew environment (4SR vs. CS and 4SS, Figure 4.1), and queens from low skew colonies engaged in antagonistic interactions when confronted with queens from high skew colonies regardless of the origin of workers (22R, 22S).

However, these preliminary results should be interpreted with caution, due to their complex nature with unequal variances and heavily skewed distributions. A visual inspection of data revealed that the variation in numbers of queens per replicate and the level of aggression among queens between replicates varies strongly. In addition, the final number of replicates per treatment varies heavily as well, and is low for several treatments (Figure 4.1). One reason for the strong variation of the data and in the number of replicates per treatment might be difficulties with the standardization of winter conditions for Reichwald-colonies, as not enough colonies could be found during the planned sampling period. Consequently, due to the complex nature of the data we did not consider further statistical analysis.

Acknowledgements

We thank Monika Haubner, Andi Sailer, Lisa Marie Braun and Thomas Seyferth for assistance during experimental preparation and observation. Supported by Deutsche Forschungsgemeinschaft (He 1623/25).

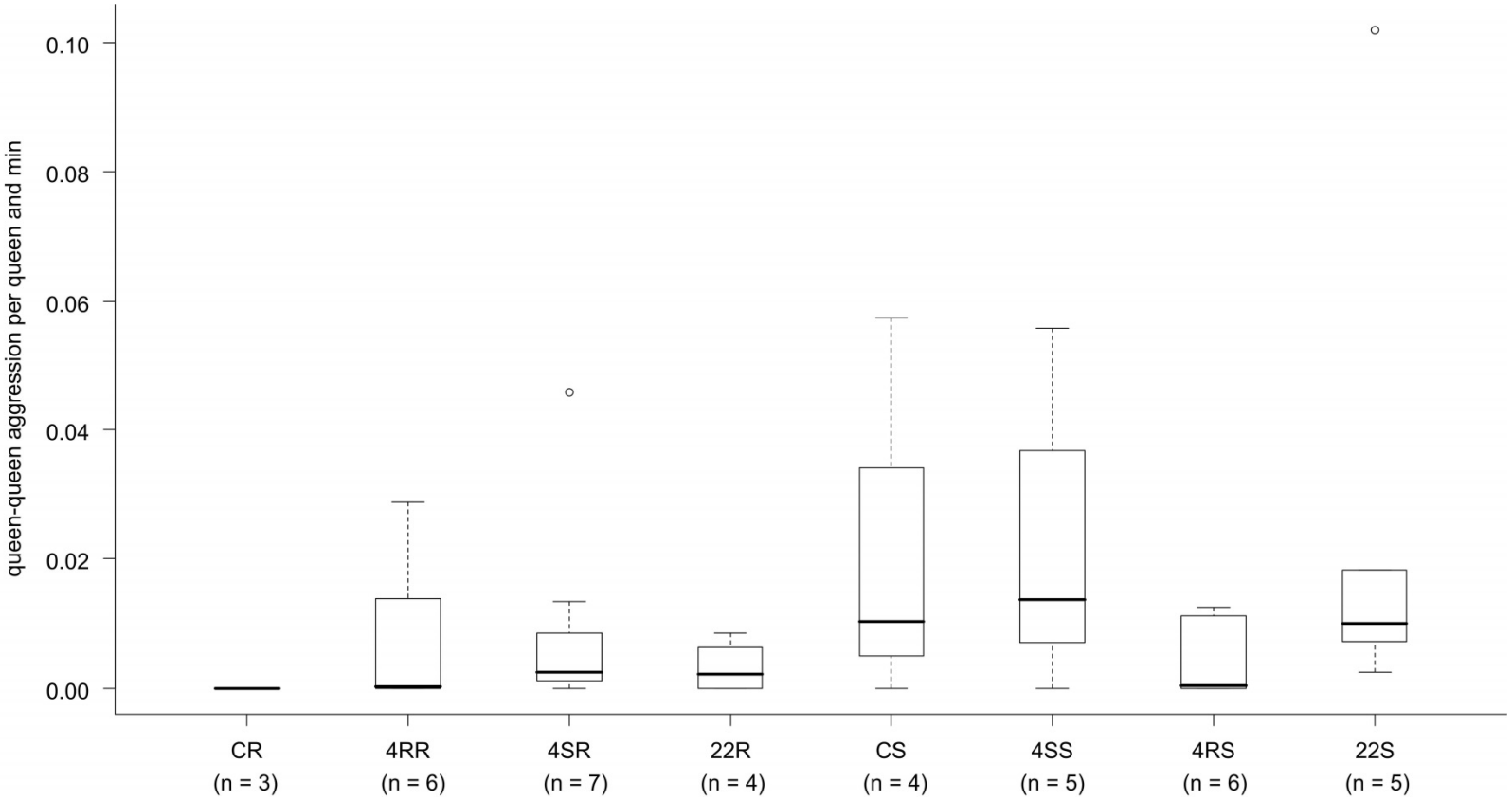

Figure 4.1 Aggression among queens in control colonies from Reichswald (CR) and S^{ra} de Albarracin (CS) and colonies in which queens were exchanged. 4RR: one queen each from four different Reichswald colonies with workers from four other Reichswald colonies, 4SR: one queen each from four different Sra de Albarracin colonies with workers from four different Reichswald colonies, 22R: one queen each from two different S^{ra} de Albarracin colonies and from two different Reichswald colonies with workers from four different Reichswald colonies and the respective combinations of queens with workers from S^{ra} de Albarracin (4SS, 4RS, 22S). *n*: no. of replicates per treatment. Open circles represent outliers.

General Discussion

In **chapter 1** we showed that aggression among queens occurs regularly in high skew colonies of *L. acervorum* from central Spain. Queen-queen aggression leads to the formation of reproductive hierarchies, in which only the top-ranking queen becomes fertile. Queens' ritualized dominance interactions (e. g., antennal boxing) together with additional aggressive or socio-positive actions of workers (i. e., attacking lower ranking queens while preferentially feeding and grooming high ranking queens), proximately underlie high reproductive skew and lead to functional monogyny. Therefore, aggressive worker policing may help to stabilize functional monogyny. In addition, our results suggest that in *L. acervorum* from central Spain comparable mechanisms of high skew formation are involved as in other functionally monogynous ants (for a detailed discussion see **chapter 1**).

In **chapter 2** and **3** we studied whether the variation of reproductive skew in *L. acervorum* represents a flexible response to changes in socio-environmental conditions or has a genetic basis only, from two different perspectives. In the first case we could show that queens from low skew colonies are principally able to react flexibly to environmental changes and adapt to the degree of reproductive skew in behavioral rather than evolutionary time. On a mechanistic level, our results suggest that an increase of queen/worker ratio and to a lesser extent food limitation elicited queen-queen antagonism in polygynous colonies from Germany similar to that underlying reproductive hierarchies in high skew populations from Spain, Japan, and Alaska. In addition, we showed that queens from manipulated colonies differed more in ovarian status than queens from control colonies. The queen-worker ratio might co-vary with ecological constraints that reflect adverse environmental conditions, which pose

high costs on dispersal and colony founding by solitary queens (Bourke & Heinze 1994 and see **chapter 1** and **2** for a detailed discussion).

In **chapter 3**, our microevolutionary analyses revealed no strong association of genetic differentiation and divergence with social organization of *L. acervorum* in SW-Europe, indicating that social phenotype is not a barrier to gene flow between low and high skew populations. Moreover, this result suggests that no strong genetic basis underlies variation in social organization of our study system. Alternatively, a lack of genome resolution (10 microsatellite loci + one mtDNA locus) might explain the observed lack of genetic differentiation between both social forms as well. Nonetheless, wherever a genetic basis for social polymorphism has been proposed (i. e. via genetic differentiation, phenotypic stability or genomic rearrangements) there is also evidence for significant mtDNA differentiation between both social forms, suggesting that a pattern of mtDNA differentiation between both social forms is an reliable indicator for a genetic basis underlying social polymorphism (for a detailed discussion see **chapter 1** and Table S3.4 therein).

In contrast to an association of genetic data with social organization, our results suggest that patterns of population structure and haplotype distribution are better explained by co-variation with climatic, ecological and historical factors, such as quaternary climate changes, oceanity-continental gradient, heterogeneous topography of Iberian Peninsula, microclimate conditions and other local effects (see **chapter 3** for a detailed discussion). Additionally, our study reveals that inner Iberian and Pyrenean populations experienced recent bottlenecks and, thus, highlights the vulnerability of *L. acvervorum* in SW-European Mountains to climate change and human induced climatic warming.

In **chapter 4** we conducted a laboratory transplantation experiment to address the question of whether variation in reproductive skew is influenced by the social environment or has a strong genetic underpinning only. Unfortunately, no clear conclusion can be drawn from the resulting data, as it is characterized by unequal variances, heavily skewed distributions

and a partial lack of expected sampled size.

Finally, the results of **chapter 2** and **3** highlight the importance of behavioral flexibility as a phenomenon involved in the adjustment of reproductive skew due to changes in environment in group living animals. Recently, behavioral plasticity (i. e. environmentally induced phenotypic variation) has gained new interest while the understanding of mechanisms underlying plasticity, in particular, has been in focus of current research (e. g., Bateson & Gluckman 2011, Kappeler et al. 2013, Snell-Rood 2013, Bateson 2014 and references therein). Interestingly, the plastic mechanisms can be categorized in different ways, such as adaptability versus conditional plasticity (sensu Bateson 2014) or activational vs. developmental plasticity (sensu Snell-Rood 2013). The flexible behavior shown by queens from low skew colonies might be plausibly identified as conditional plasticity (i. e., as a plastic response evolved to repeated challenges from the environment, Bateson 2014). Alternatively, the same behavior can be rephrased in terms of activational plasticity (i. e., a differential activation of an underlying network in different environments by which an individual can change its phenotype throughout life, Snell-Rood 2013).

In addition, theory predicts that in an evolving metapopulation with migration (i. e., where populations experience spatial environmental heterogeneity with multidirectional gene flow between patches) plasticity is favored over adaptive genetic divergence between populations (Sultan & Spencer 2002, see also Moran 1992, Roff 1996, Crispo 2008 and references therein). While our results of **chapter 2** and **3** independently from each other seem to support this prediction, additional future research is needed to confirm or reject these findings. This future work could, for example, include the repetition of the experiment done in **chapter 2**, with colonies from high skew populations of *L. acervorum* by adding workers or overfeeding colonies. Similarly, our microevolutionary study in **chapter 3** should be supplemented by cross-fostering experiments, breeding pedigrees, genome scans or functional

genomic analyses to clarify definitively to which extent social organization in *L. acervorum* is under genetic control or represents environmentally induced plasticity.

REFERENCES

Atlas Climático Digital de Aragón [http://www.opengis.uab.es/wms/Aragon/index.htm]

Bargum K, Helanterä H, Sundström L (2007) Genetic population structure, queen supersedure and social polymorphism in a social Hymenoptera. J Evol Biol 20: 1351-1360.

Barth MB, Moritz RFA, Pirk CWW, Kraus FB (2013) Male-biased dispersal promotes large scale gene flow in a subterranean army ant, *Dorylus (Typhlopone) fulvus*. Popul Ecol 55: 523-533. doi:10.1007/s10144-013-0383-4

Bateson P (2014) Why are individuals so different from each other? Heredity November 19, 2014; (doi:10.1038/hdy.2014.103).

Bateson P, Gluckman P (2011). Plasticity, Robustness, Development and Evolution. Cambridge University Press: Cambridge, UK, 156pp.

Bennett KD (1997) Evolution and ecology: the pace of life. Cambridge University Press, Cambridge, 241pp.

Bhargava A, Fuentes FF (2010) Mutational dynamics of microsatellites. Mol Biotechnol 44: 250-266.

Birks HJB, Willis KJ (2008) Alpines, trees, and refugia in Europe. Plant Ecol Divers 1: 147-160.

Blanco-Fontao B, Fernández-Gil A, Obeso JR, Quevedo M (2010) Diet and habitat selection in Cantabrian Capercaillie (*Tetrao urogallus cantabricus*): ecological differentiation of a rear-edge population. J Ornithol 151: 269-277.

Bourke AFG (1988) Worker reproduction in the higher eusocial Hymenoptera. Q Rev Biol 63: 291-311.

Bourke AFG (1991) Queen behaviour, reproduction and egg cannibalism in multiple- queen colonies of the ant *Leptothorax acervorum*. Anim Behav 42: 295-310.

Bourke AFG (1994) Indiscriminate egg cannibalism and reproductive skew in a multiple-queen ant. Proc R Soc Lond B 255: 55-59.

Bourke AFG, Heinze J (1994) The ecology of communal breeding: the case of multiply-queened leptothoracine ants. Phil Trans R Soc Lond B 345: 359-372.

Bourke AFG, Green HA, Bruford MW (1997) Parentage, reproductive skew and queen turn-over in a multiple-queen ant analysed with microsatellites. Proc R Soc Lond B 264: 277-283.

Brandt M, Fischer-Blass B, Heinze J, Foitzik S (2007) Population structure and co-evolution between social parasites and their hosts. Mol Ecol 16: 2063-2078.

Buschinger A (1968) Mono- und Polygynie bei Arten der Gattung *Leptothorax* Mayr (Hymenoptera Formicidae). Insectes Soc 15: 217-226.

Buschinger A (1973) The role of daily temperature rhythms in brood development of ants of the tribe Leptothoracini (Hymenoptera; Formicidae). In: Wieser W (ed) Effects of temperature on ectothermic organisms. Springer, Berlin, pp 229-232.

Buschinger A (1974a) Experimente und Beobachtungen zur Gründung und Entwicklung neuer Sozietäten der sklavenhaltenden Ameise *Harpagoxenus sublaevis* (Nyl.). Insectes Soc 21: 381-406.

Buschinger A, (1974b) Polymorphismus und Kastendetermination im Ameisentribus Leptothoracini. In: Schmidt GH (ed) Sozialpolymorphismus bei Insekten. Wiss. Verlagsges. mbH, Stuttgart, pp 604-623.

Buschinger A, Alloway TM (1978) Caste polymorphism in *Harpagoxenus canadensis* M. R. Smith (Hym. Formicidae). Insectes Soc 25: 339-350.

Carrión JS, Fernández S, González-Sampériz P, Gil-Romera G, Badal E, Carrión-Marco Y, et al. (2010) Expected trends and surprises in the Lateglacial and Holocene vegetation history of the Iberian Peninsula and Balearic Islands. Review of Palaeobotany and Palynology 162: 458-475.

Cassel-Lundhagen A (2010) Peripheral Relict Populations of Widespread Species; Evolutionary Hotspots or Just More of the Same? In: Habel JC, Assmann T (eds) Relict species. Springer, Berlin, pp 267-275.

Chapuisat M, Bocherens S, Rosset H (2004) Variable queen number in ant colonies: no impact on queen turnover, inbreeding, and population genetic differentiation in the ant *Formica selysi*. Evolution 58: 1064-1072.

Chen YP, Vinson SB (2000) Effects of Queen Attractiveness to Workers on the Queen Nutritional Status and Egg Production in the Polygynous *Solenopsis invicta* (Hymenoptera: Formicidae). Ann Entomol Soc Am 93: 295-302.

Choe JC, Perlman DL (1997) Social conflict and cooperation among founding queens in ants (Hymenoptera: Formicidae). In: Choe JC, Crespi BJ (eds) The Evolution of Social Behavior in Insects and Arachnids. Cambridge University Press, Cambridge, pp 392-406.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657-1659.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144: 2001-2014.

Crawford RMM (2005) Trees by the sea: advantages and disadvantages of oceanic climates. Proc R Irish Acad 105B: 129-139.

Crispo E (2008) Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. J Evol Biol 21: 1460-1469.

Czechowski W, Radchenko A, Czechowska W, Vepsäläinen K (2012) The ants of Poland with reference to the myrmecofauna of Europe. Fauna Poloniae 4. - Natura Optima Dux Foundation, Warsaw, 496 pp.

Davis BAS, Stevenson AC (2007) The 8.2 ka event and early-mid Holocene forests, fires and flooding in the Central Ebro Desert, NE Spain. Quaternary Science Reviews 26: 1695-1712.

Drees C, Matern A, Oheimb G von, Reimann T, Assmann T (2010) Multiple glacial refuges of unwinged ground beetles in Europe: molecular data support classical Phylogeographic models. In: Habel JC, Assmann T (eds) Relict species. Springer, Berlin, pp 199-215.

Duriez O, Sachet JM, Ménoni E, Pidancier N, Miquel C, Taberlet P (2007) Phylogeography of the capercaillie in Eurasia: what is the conservation status in the Pyrenees and Cantabrian Mounts? Conserv Genet 8 513-526.

Earl DA, vonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4: 359-361 doi: 10.1007/s12686-011-9548-7

Eckert CG, Samis KE, Lougheed SC (2008) Genetic variation across species' geographical ranges: the central–marginal hypothesis and beyond. Mol Ecol 17: 1170-1188.

Evans JD (1993) Parentage analysis in ant colonies using simple sequence repeat. Mol Ecol 2: 393-397.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611-2620.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 10: 564-567.

Felke M, Buschinger A (1999) Social organization, reproductive behavior and ecology of *Leptothorax acervorum* (Hymenoptera, Formicidae) from the Sierra de Albarracin in central Spain. Insectes Sociaux 46: 84-91. (doi:10.1007/s000400050117)

Field J, Paxton RJ, Soro A, Bridge C (2010) Cryptic plasticity underlies a major evolutionary transition. Curr Biol 20: 2028-2031.

Fink S, Excoffier L, Heckel G (2004) Mitochondrial gene diversity in the common vole *Microtus arvalis* shaped by historical divergence and local adaptations. Mol Ecol 13: 3501-3514.

Foitzik S, Haberl M, Gadau J, Heinze J (1997) Mating frequency of *Leptothorax nylanderi* ant queens determined by microsatellite analysis. Insectes Soc 44: 219-227.

Foitzik S, Bauer S, Laurent S, Pennings PS (2009) Genetic diversity, population structure and sex-biased dispersal in three co-evolving species. Journal of Evolutionary Biology 22: 2470-2480.

Forsyth A (1980) Worker control of queen density in Hymenopteran societies. Am Nat 116: 895-898.

Francoeur A (1983) The ant fauna near the tree-line in northern Québec (Formicidae, Hymenoptera). Nordicana 47: 177-180.

Franks NR, Scovell E (1983) Dominance and reproductive success among slave-making worker ants. Nature 304: 724-725.

Garrick RC, Caccone A, Sunnucks P (2010) Inference of population history by coupling exploratory and model-driven phylogeographic analyses. Int J Mol Sci 11: 1190-1227.

Garza JC (2011) Critical M. [http://swfsc.noaa.gov/textblock.aspx?Division=FED&id=3298]

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. Mol Ecol 10: 305-318.

Gilchrist JS, Otali E, Mwanguhya F (2004) Why breed communally? Factors affecting fecundity in a communal breeding mammal: the banded mongoose (*Mungos mungo*). Behav Ecol Sociobiol 57: 119-131.

Gill RJ, Arce A, Keller L, Hammond RL (2009) Polymorphic social organization in an ant. Proceedings of the Royal Society B 276: 4423-4431.

Gill RJ, Hammond RL (2011) Workers influence royal reproduction. Proc R Soc Lond B 278: 1524-1531.

Gomez A, Lunt DH (2007) Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula. In: Weiss S, Ferrand N (eds) Phylogeography of Southern European Refugia. Springer, Berlin, pp 155-188.

Gotzek D, Ross KG (2007) Genetic regulation of colony social organization in fire ants: an integrative overview. Q Rev Biol 82: 201-266.

Goudet J (1995) Fstat version 1.2: a computer program to calculate Fstatistics. Journal of Heredity 86: 485-486.

Habel JC, Assmann T, Schmitt T, Avise JC (2010) Relict Species: From Past to Future. In: Habel JC, Assmann T (eds) Relict species. Springer, Berlin, pp 1-5.

Hager R, Jones CB (2009) Reproductive Skew in Vertebrates: Proximate and Ultimate Causes. Cambridge University Press, Cambridge, 523pp.

Hall TA (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Hamaguchi K, Ito Y (1993) GT Dinucleotide Repeat Polymorphisms in a Polygynous Ant, *Leptothorax spinosior* and Their Use for Measurement of Relatedness. Naturwissenschaften 80: 179-181.

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Palaeontological statistics software package for education and data analysis. Palaeontol Electron 4: 9.

Hammond RL, Keller L (2004) Conflict over male parentage in social insects. *PloS Biol* 2: e248.

Hammond RL, Bruford MW, Bourke AFG (2006) A test of reproductive skew models in a field population of a multiple-queen ant. Behav Ecol Sociobiol 61: 265-275.

Hammouti N, Schmitt T, Seitz A, Kosuch J, Veith M (2010) Combining mitochondrial and nuclear evidences: a refined evolutionary history of *Erebia medusa* (Lepidoptera: Nymphalidae: Satyrinae) in Central Europe based on the CO1 gene. J Zool Syst Evol Res 48: 115-125.

Heino M, Hanski I (2001) Evolution of migration rate in a spatially realistic metapopulation model. American Naturalist 157: 495-511.

Heinze J (1992) Ecological correlates of functional monogyny and queen dominance in leptothoracine ants. In: Billen J (ed) Biology and Evolution of Social Insects. Leuven University Press, Leuven, pp 25-33.

Heinze J (2004) Reproductive conflict in insect societies. Adv Stud Behav 34: 1-57.

Heinze J (2010) Conflict and conflict resolution in social insects. In: Kappeler P (ed) Animal behaviour: Evolution and mechanisms. Springer, Berlin, pp 151-178.

Heinze J, Smith TA (1990) Dominance and fertility in a functionally monogynous ant. Behav Ecol Sociobiol 27: 1-10.

Heinze J, Ortius D (1991) Social organization of *Leptothorax acervorum* from Alaska (Hymenoptera: Formicidae). Psyche 98: 227-240.

Heinze J, Lipski N, Hölldobler B (1992) Reproductive competition in colonies of the ant *Leptothorax gredleri*. Ethology 90: 265-278.

Heinze J, Gübitz T, Errard C, Lenoir A, Hölldobler B (1993) Reproductive competition and colony fragmentation in the guest ant *Formicoxenus provancheri*. Experientia 49: 814-816.

Heinze J, Hölldobler B, Peeters C (1994) Cooperation and conflict in ant societies. Naturwissenschaften 81: 489-497.

Heinze J, Lipski N, Hölldobler B, Bourke AFG (1995a) Geographic variation in the social and genetic structure of the ant, *Leptothorax acervorum*. Zoology 98: 127-135.

Heinze J, Lipski N, Schlehmeyer K, Hölldobler B (1995b) Colony structure and reproduction in the ant *Leptothorax acervorum*. Behav Ecol 6: 359-367.

Heinze J, Stahl M, Hölldobler B (1996) Ecophysiology of hibernation in boreal *Leptothorax* ants (Hymenoptera: Formicidae). Écoscience 3: 429-435.

Heinze J, Oberstadt B, Tentschert J, Hölldobler B, Bestmann HJ (1998) Colony specificity of Dufour gland secretions in a functionally monogynous ant. Chemoecology 8: 169-174.

Heinze J, d'Ettorre P (2009) Honest and dishonest communication in social Hymenoptera. J exp Biol 212: 1775-1779.

Helms KR, Helms Cahan S (2012) Large scale regional variation in cooperation and conflict among queens of the desert ant *Messor pergandei*. Anim Behav 84: 499-507.

Helms KR, Newman NJ, Helms Cahan S (2013) Regional variation in queen and worker aggression in incipient colonies of the desert ant *Messor pergandei*. Behav Ecol Sociobiol 67: 1563-1573.

Hemelrijk CK, Wantia J, Gygax L (2005) The construction of dominance order: comparing performance of five methods using an individual-based model. Behaviour 142:1037-1058.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. Biol J Linn Soc 58: 247-276.

Hewitt GM (2001) Speciation, hybrid zones and phylogeography – or seeing genes in space and time. Mol Ecol 10: 537-549.

Hewitt GM (2004) The structure of biodiversity – insights from molecular phylogeography. Front Zool 1: 4.

Hewitt GM (2011a) Quaternary phylogeography: the roots of hybrid zones. Genetica 139: 617-638.

Hewitt GM (2011b) Mediterranean Peninsulas: The Evolution of Hotspots. In: Zachos FE, Habel JC (eds) Biodiversity Hotspots. Springer, Berlin, pp 123-147.

Holm S (1979) A simple sequentially rejective multiple test procedure. Scand J Stat 6: 65–70.

Huck S, Büdel B, Kadereit JW, Printzen C (2009) Range-wide phylogeography of the European temperate montane herbaceous plant *Meum athamanticum* Jacq.: evidence for periglacial persistence. J Biogeogr 36: 1588-1599.

Ito F (1990) Functional monogyny of *Leptothorax acervorum* in northern Japan. Psyche 97: 203-211.

Ito F (2005) Mechanisms regulating functional monogyny in a Japanese population of *Leptothorax acervorum* (Hymenoptera, Formicidae): dominance hierarchy and preferential egg cannibalism. Belg J Zool 135: 3-8.

Jamieson IG (1997) Testing reproductive skew models in a communally breeding bird, the pukeko, *Porphyrio porphyrio*. Proc R Soc Lond B 264: 335-340.

Johnstone RA (2000) Models of reproductive skew: a review and synthesis. Ethology 106: 5-26.

Jost L (2008) G_{ST} and its relatives do not measure differentiation. Mol Ecol 17: 4015-4026.

Kappeler PM, Barrett L, Blumstein DT, Clutton-Brock TH (2013) Constraints and flexibility in mammalian social behaviour: introduction and synthesis. Phil. Trans. R. Soc. B 368: 20120337. (doi:10.1098/rstb.2012.0337).

Keller L, Reeve HK (1994) Partitioning of reproduction in animal societies. Trends Ecol Evol 9: 98-102.

Kokko H (2003) Are reproductive skew models evolutionarily stable? Proc R Soc Lond B 270: 265-270.

Kropf M, Comes HP, Kadereit JW (2012) Past, present and future of mountain species of the French Massif Central–the case of *Soldanella alpina* L. subsp. *alpina* (Primulaceae) and a review of other plant and animal studies. Journal of Biogeography 39: 799-812.

Kutsukake N, Nunn CL (2006) Comparative tests of reproductive skew in male primates: the roles of demographic factors and incomplete control. Behav Ecol Sociobiol 60: 695–706.

Langella O (1999) Populations 1.2.31: a population genetic software. Roscoff, France. UPR9034: Available at http://bioinformatics.org/~tryphon/populations: Centre National de la Recherche Scientifique, Evolution, Génomes et Spéciation.

Larkin MA, Blackshields G, Brown NP, Chenna R, Mc-Gettigan PA, McWilliam H, et al. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947-2948.

Leppänen J, Vepsäläinen K, Savolainen R (2011) Phylogeography of the ant *Myrmica rubra* and its inquiline social parasite. Ecology and Evolution 1: 46-62.

Leppänen J, Vepsäläinen K, Anthoni H, Savolainen R (2013) Comparative Phylogeography of the ants *Myrmica ruginodis* and *Myrmica rubra*. J Biogeogr 40: 479-491.

Librado P, Rozas J (2009) DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451-1452.

Linklater WL (2000) Adaptive explanation in socio-ecology: lessons from the Equidae. Biol Rev 75: 1-20.

Lipski N, Heinze J, Hölldobler B (1992) Social organization of three European *Leptothorax* species (Hym: Formicidae). In: Billen J (ed) Biology and Evolution of Social Insects. Leuven University Press, Leuven, pp. 287-290.

Magrath RD, Heinsohn RG (2000) Reproductive skew in birds: models, problems and prospects. J Avian Biol 31: 247-258.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. Cancer Research 27: 209-220.

Martin P, Bateson P (2007) Measuring behaviour: an introductory guide. Cambridge University Press, Cambridge 184pp.

Monnin T, Ratnieks FLW (2001) Policing in queenless ponerine ants. *Behav Ecol Sociobiol* 50: 97-108.

Monnin T, Ratnieks FLW, Jones GR, Beard R (2002) Pretender punishment induced by chemical signalling in a queenless ant. Nature 419: 61-65.

Moran NA (1992) The evolutionary maintenance of alternative phenotypes. Amer Nat 139: 249-278.

Nei M, Chesser RK (1983) Estimation of fixation indices and gene diversities. Ann Hum Genet 47: 253-259.

Nieto Feliner G (2011) Southern European glacial refugia: A tale of tales. Taxon 60: 365-372.

Nonacs P (2000) Measuring and using skew in the study of social behavior and evolution. Am Nat 156: 577-589.

Nonacs P, Hager R (2011) The past, present and future of reproductive skew theory and experiments. Biol Rev 86: 271-298.

Ortius D, Heinze J (1999) Fertility signaling in queens of a North American ant. Behav Ecol Sociobiol 45: 151-159.

Ostfeld RS (1990) The ecology of territoriality in small mammals. Trends Ecol Evol 5: 411-415.

Overson R, Gadau J, Clark RM, Pratt SC, Fewell JH (2013) Behavioral transitions with the evolution of cooperative nest founding by harvester ant queens. Behav Ecol Sociobiol: DOI 10.1007/s00265-013-1618-2.

Pardi L (1946) Ricerche sui Polistini. VII. La "dominazione" e il ciclo ovarico annuale in *Polistes gallicus* L. Boll Inst Entomol "Guido Grandi" Univ Stud Bologna 15: 25-84.

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. Bioinformatics 28: 2537-2539.

Peery MZ, Kirby R, Reid BN, Stoelting R, Doucet-Beer E, Robinson S, et al. (2012) Reliability of genetic bottleneck tests for detecting recent population declines. Mol. Ecol. 21: 3403-3418. doi:10.1111/j.1365-294X. 2012.05635.x.

Pérez-Obiol R, Jalut G, Julià R, Pèlachs A, Iriarte MJ, Otto T, Hernàndez-Beloqui B (2011) Mid-Holocene vegetation and climatic history of the Iberian Peninsula. Holocene 21:75-93.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity 90: 502-503.

Pons O, Petit RJ (1996) Measuring and testing genetic differentiation with ordered versus unordered alleles. Genetics 144: 1237-1245.

Port M, Kappeler PM (2010) The utility of reproductive skew models in the study of male primates, a critical evaluation. Evolutionary Anthropology 19: 46-56.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155: 945-959.

Provan J, Bennett KD (2008) Phylogeographic insights into cryptic glacial refugia. Trends Ecol Evol 23: 564-571.

Queller DC, Goodnight KF (1989) Estimating relatedness using genetic markers. Evolution 43: 258-275.

R Development Core Team (2014) R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.

Ratnieks FLW (1988) Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. Am Nat 132: 217-236.

Ratnieks FLW, Foster KR, Wenseleers T (2006) Conflict resolution in insect societies. Annu Rev Entomol 51: 581-608.

Reeve HK, Ratnieks FLW (1993) Queen–queen conflicts in polygynous societies: mutual tolerance and reproductive skews. In: Keller L, editor. Queen Number and Sociality in Insects. Oxford: Oxford University Press, pp. 45-85.

Reeve HK (2000) A transactional theory of within-group conflict. Am Nat 155: 365-382

Reeve HK, Keller L (2001) Tests of reproductive-skew models in social insects. Annu Rev Entomol 46: 347-385.

Reeve HK, Shen SF (2013) Unity and disunity in the search for a unified reproductive skew theory. Anim Behav 85: 1137-1144.

Rodríguez Muñoz R, Mirol PM, Segelbacher G, Fernández A, Tregenza T (2007) Genetic differentiation of an endangered capercaillie (*Tetrao urogallus*) population at the Southern edge of the species range. Conserv Genet 8: 659-670.

Rogers AR, Harpending H (1992) Population growth makes waves in the distribution of pairwise genetic differences. Mol Biol Evol 9: 552-569.

Roff DA (1996) The evolution of threshold traits in animals. Q Rev Biol 71: 3-35.

Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. Mol Ecol Notes 4: 137-138.

Ross KG, Keller L (1998) Genetic control of social organization in an ant. Proc Natl Acad Sci USA 95: 14232-14237.

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Res 8: 103-106.

Rubiales JM, García-Amorena I, García Álvarez S, Gómez Manzaneque F (2008) The Late Holocene extinction of *Pinus sylvestris* in the western Cantabrian Range (Spain). J. Biogeogr. 35: 1840–1850.

Rubiales JM, García-Amorena I, Hernández L, Génova M, Martínez F, Gómez Manzaneque F, Morla C (2010) Late Quaternary dynamics of pinewoods in the Iberian mountains. Review of Palaeobotany and Palynology 162: 476-491.

Rubiales JM, Morales-Molino C, García S, García-Antón M (2012) Negative responses of highland pines to anthropogenic activities in inland Spain: a palaeoecological perspective. Vegetation History and Archaeobotany 21: 397-412.

Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B (2000) Artemis: sequence visualization and annotation. Bioinformatics 16: 944-945.

Rüppell O, Schäffler L, Hölldobler B (2002) Lack of plasticity in the behaviour of queens of the ant *Leptothorax rugatulus* Emery (Formicidae: Hymenoptera). J Insect Behav 15: 447-454.

Salomon M, Mayntz D, Lubin Y (2008) Colony nutrition skews reproduction in a social spider. Behav Ecol 19: 605-611.

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sanetra M, Crozier RH (2003) Patterns of population subdivision and gene flow in the ant *Nothomyrmecia macrops* reflected in microsatellite and mitochondrial DNA markers. Molecular Ecology 12: 2281-2295.

Schmalhausen II (1986) Factors of Evolution: The Theory of Stabilizing Selection. University of Chicago Press, Chicago, 327pp.

Schmitt T (2007) Molecular biogeography of Europe: Pleistocene cycles and postglacial trends. Front Zool 4: 11.

Schmitt T (2009) Biogeographical and evolutionary importance of the European high mountain systems. Front Zool 6: 9.

Schmitt T, Seitz A (2001) Intraspecific allozymatic differentiation reveals the glacial refugia and the postglacial expansions of European *Erebia medusa* (Lepidoptera: Nymphalidae). Biol J Linn Soc 74: 429-458.

Schmitt T, Hewitt GM, Müller P (2006) Disjunct distributions during glacial and interglacial periods in mountain butterflies: *Erebia epiphron* as an example. J Evol Biol 2006 19:108-113.

Schmitt T, Varga Z (2012) Extra-Mediterranean refugia: The rule and not the exception? Frontiers in Zoology 9: 22.

Schwander T, Lo N, Beekman M, Oldroyd BP, Keller L (2010) Nature versus nurture in social insect caste differentiation. Trends Ecol Evol 25: 275-282.

Seifert B (2007) Ameisen Mittel- und Nordeuropas. Lutra, Tauer, 368pp.

Sherman PW, Lacey EA, Reeve HK, Keller L (1995) The eusocialitiy continuum. Behav Ecol 6: 102-108.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved PCR primers. Ann Entomolo Soc Am 87: 651-701.

Snell-Rood EC (2013) An overview of the evolutioary causes and consequences of behavioural plasticity. Anim Behav 85: 1004-1011.

Soare TW, Kumar A, Naish KA, O`Donnelly S (2014) Genetic evidence for landscape effects on dispersal in the army ant *Eciton burchellii*. Molecular Ecology 23: 96-109.

Sokal RR, Rohlf FJ (1995) Biometry: the Principles and Practices of Statistics in Biological Research. W.H. Freeman, New York, 887pp.

Sommeijer MJ, Van Veen JW (1990) The polygyny of *Myrmica rubra*: selective oophagy and trophallaxis as mechanisms of reproductive dominance. Entomol Exp Appl 56: 229-239.

Song H, Buhay JE, Whiting MF, Crandall KA (2008) Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. Proc Natl Acad Sci USA 105: 13486-13491.

Soro A, Field J, Bridge C, Cardinal SC, Paxton RJ (2010) Genetic differentiation across the social transition in a socially polymorphic sweat bee, *Halictus rubicundus*. Mol Ecol 19: 3351-3363.

Stewart JR, Lister AM (2001) Cryptic northern refugia and the origins of the modern biota. Trends Ecol Evol 16: 608-613.

Stille M, Stille B, Douwes P (1991) Polygyny, relatedness and nest founding in the polygynous myrmicine ant *Leptothorax acervorum*. (Hymenoptera: Formicidae). Behav Ecol Sociobiol 28: 91-96.

Storch I (2007) Grouse: status survey and conservation action plan 2006–2010. IUCN, Gland, Switzerland and Cambridge, UK, and the World Pheasant Association, Fordingbridge, UK.

Strassmann JE, Queller DC (2007) Insect societies as divided organisms: The complexities of purpose and cross-purpose. Proc Natl Acad Sci USA 104: 8619-8626.

Suefuji M, Trindl A, Heinze J (2011) Characterization of 13 microsatellite markers for the threatened, slave-making ant *Myrmoxenus ravouxi* (Formicidae: Myrmicinae). Conserv Genet Resour 3: 229-331.

Sultan SE, Spencer HG (2002) Metapopulation structure favors plasticity over local adaptation. Amer Nat 160: 271-283.

Sundström L, Seppä P, Pamilo P (2005) Genetic population structure and dispersal patterns in Formica ants – a review. Annales Zoologici Fennici 42: 163-177.

Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF (1998) Comparative phylogeography and postglacial colonization routes in Europe. Molecular Ecology 7: 453-464.

Thuiller W, Lavorei S, Araújo MB, Sykes MT, Prentice IC (2005) Climate change threats to plant diversity in Europe. Proc Natl Acad Sci USA 102: 8245-8250.

Trettin J, Haubner M, Buschinger A, Heinze J (2011) Queen dominance and worker policing control reproduction in a threatened ant. BMC Ecology 11: 21.

Trettin J, Seyferth T, Heinze J (2014) Behavioral Plasticity in Ant Queens: Environmental Manipulation Induces Aggression among Normally Peaceful Queens in the Socially Polymorphic Ant *Leptothorax acervorum*. PLoS ONE 9: e95153. doi:10.1371/journal.pone.0095153.

Trubenová B, Hager R (2012) Reproductive Skew Theory. eLS. DOI: 10.1002/9780470015902.a0023661.

Tzedakis PC, Emerson BC, Hewitt GM (2013) Cryptic or mystic? Glacial tree refugia in northern Europe. Trends Ecol Evol 28: 696-704.

Ursenbacher S, Carlsson M, Helfer V, Tegelström H, Fumagalli L (2006) Phylogeography and Pleistocene refugia of the adder (*Vipera berus*) as inferred from mitochondrial DNA sequence data. Mol Ecol 15: 3425-3437.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4: 535-538.

Vehrencamp SL (1983) A model for the evolution of despotic versus egalitarian societies. Anim Behav 31: 667-682.

Wang J, Wurm Y, Nipitwattanaphon M, Riba-Grognuz O, Huang YC, Shoemaker D, Keller L (2013). A Y-like social chromosome causes alternative colony organization in fire ants. Nature 493: 664-668.

Weir BS (1996) Genetic data analysis II: Methods for discrete population genetic data. Sinauer Associates, Inc. Publishers, Sunderland, Massachusetts, 445pp.

Wenseleers T, Ratnieks FLW (2006) Enforced altruism in insect societies. Nature 444: 50.

West-Eberhard MJ (2003) Developmental Plasticity and Evolution. Oxford University Press, Oxford, 794pp.

Wilson EO (1971) The Insect Societies. Harvard University Press, Cambridge, Massachusetts. 548pp.

Yamauchi K, Kawase N (1992) Pheromonal manipulation of workers by a fighting male to kill his rival males in the ant *Cardiocondyla wroughtonii*. Naturwissenschaften 79: 274-276.

Yamauchi K, Ishida Y, Hashim R, Heinze J (2007) Queen-queen competition and reproductive skew in a *Cardiocondyla* ant. Insectes Soc 54: 268-274.

APPENDIX

(In Figures and Tables, the first number refers to the chapter containing them and the second number refers to the order of Figures and Tables within a chapter.)

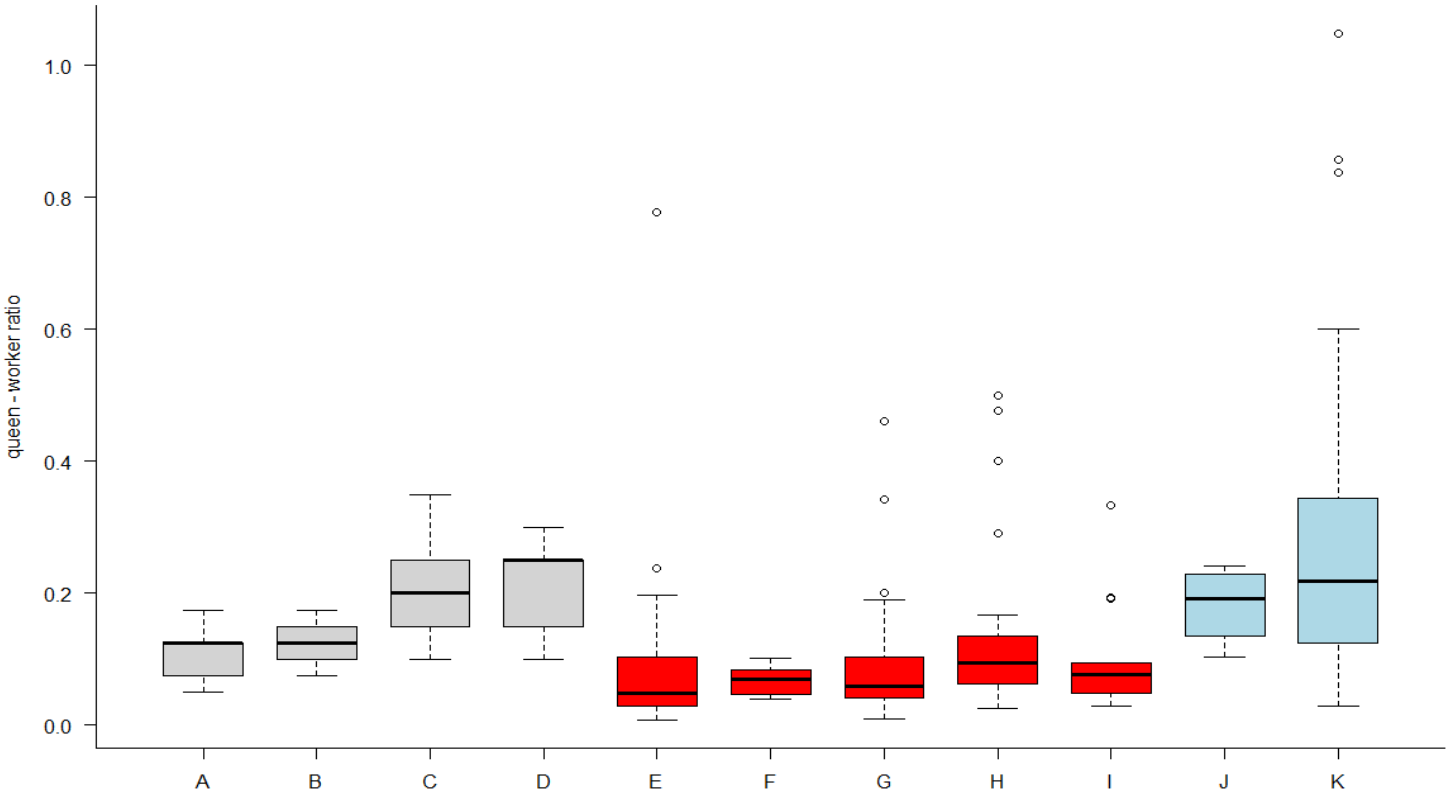

Figure S2.1 Queen-worker ratios of experimental treatments from this study (grey) as well as natural queen-worker ratios from several low skew (red) and high skew (blue) populations. (A: controls, B: food reduction, C: worker reduction, D: both treatments (FW), E – K: correspond to references in Table S2.1; E = 1, F = 2, G & H & I = 3, J = 4, K = 5 & 6).

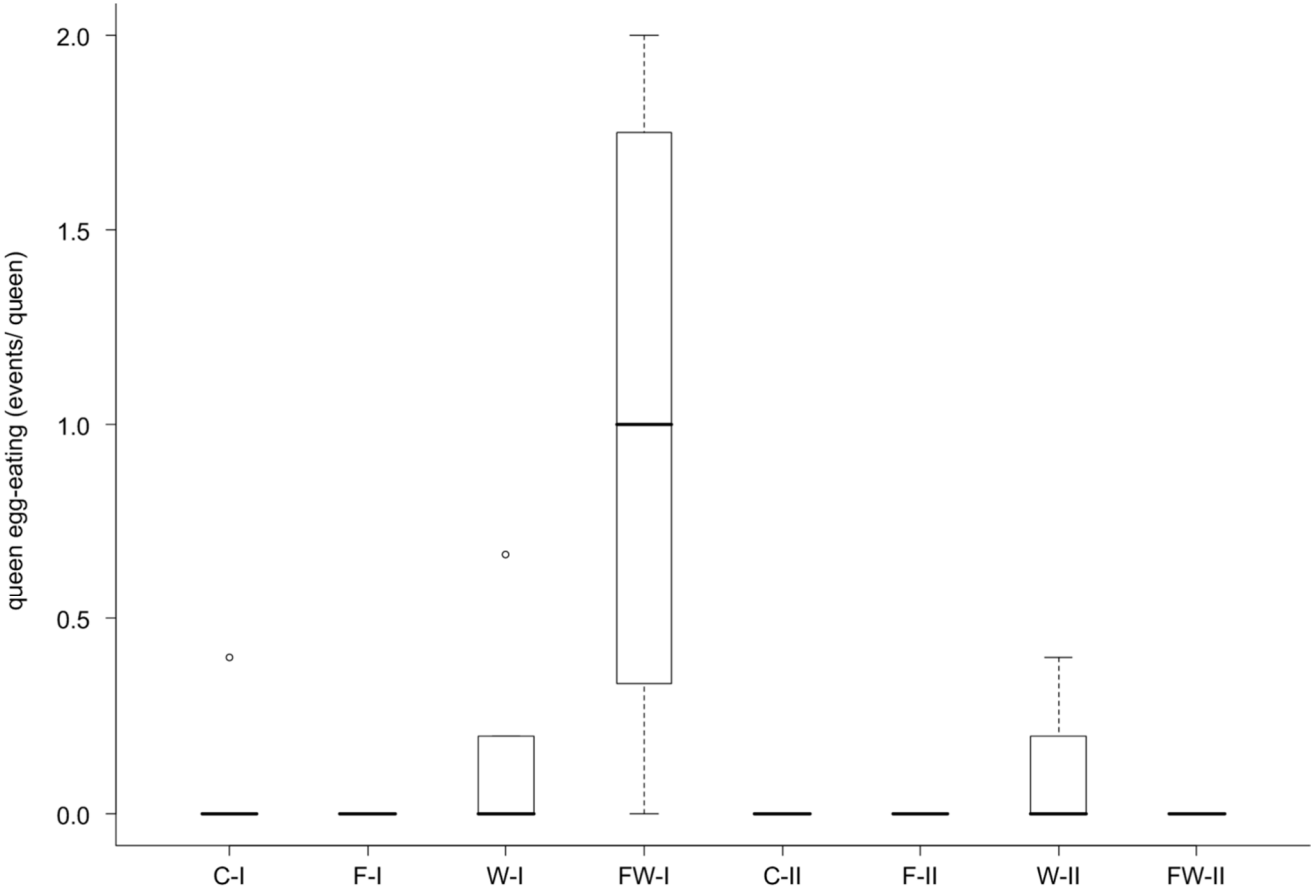

Figure S2.2 Frequency of egg eating (observations per queen during the total observation period, median, quartiles, range) in colonies of the ant *Leptothorax acervorum* from the low-skew population in Nürnberger Reichswald. Individual colonies were subjected to different types of stress (food reduction F, worker reduction W, or both FW) or left unmanipulated (control C) in two different seasons (July, I, and September, II). Outliers are indicated as circles.

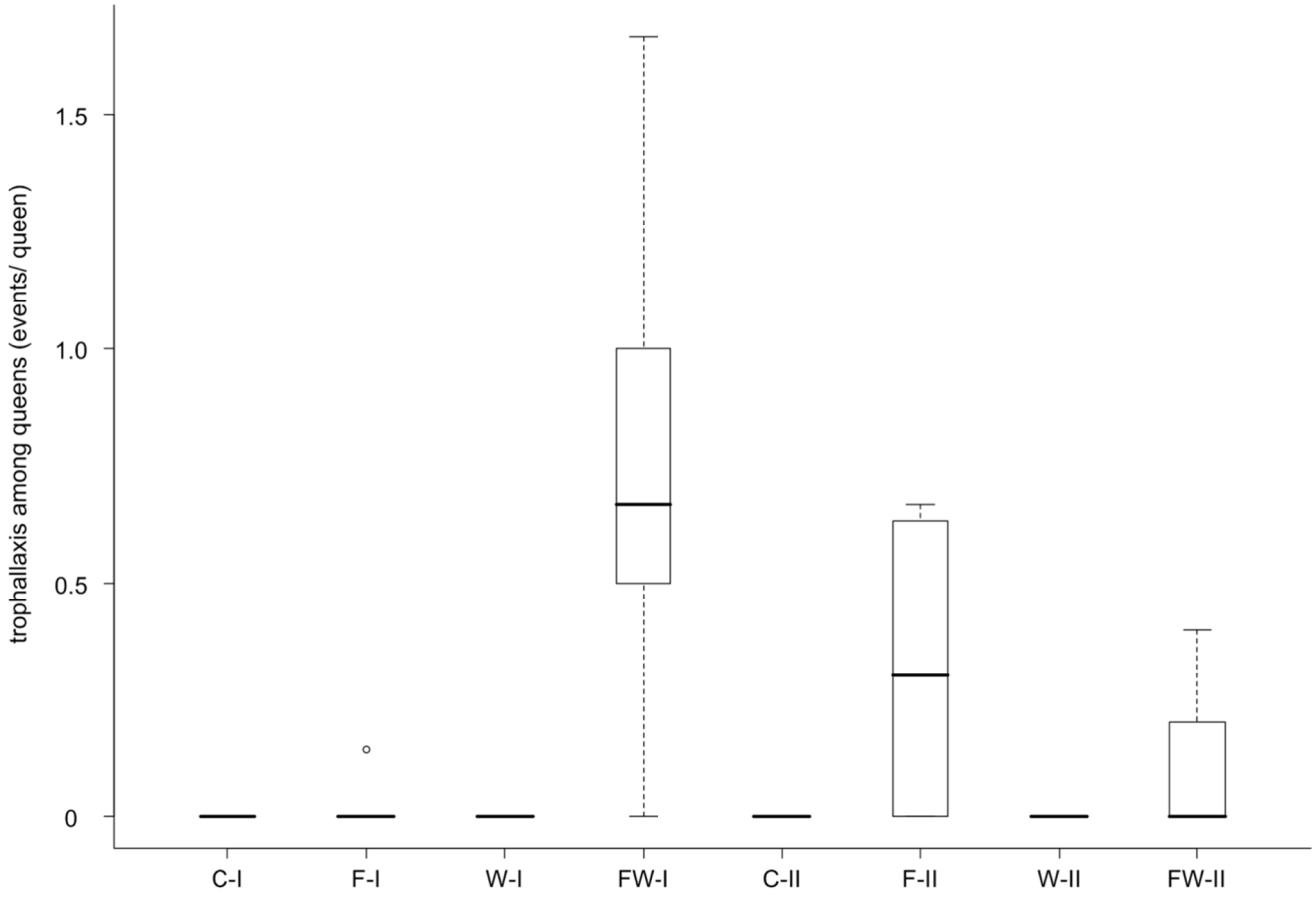

Figure S2.3 Frequency of trophallaxis, i.e., food exchange, among queens (observations per queen during the total observation period, median, quartiles, range) of the ant *Leptothorax acervorum* from the low-skew population in Nürnberger Reichswald. Individual colonies were subjected to different types of stress (food reduction F, worker reduction W, or both FW) or left unmanipulated (control C) in two different seasons (July, I, and September, II). Outliers are indicated as circles.

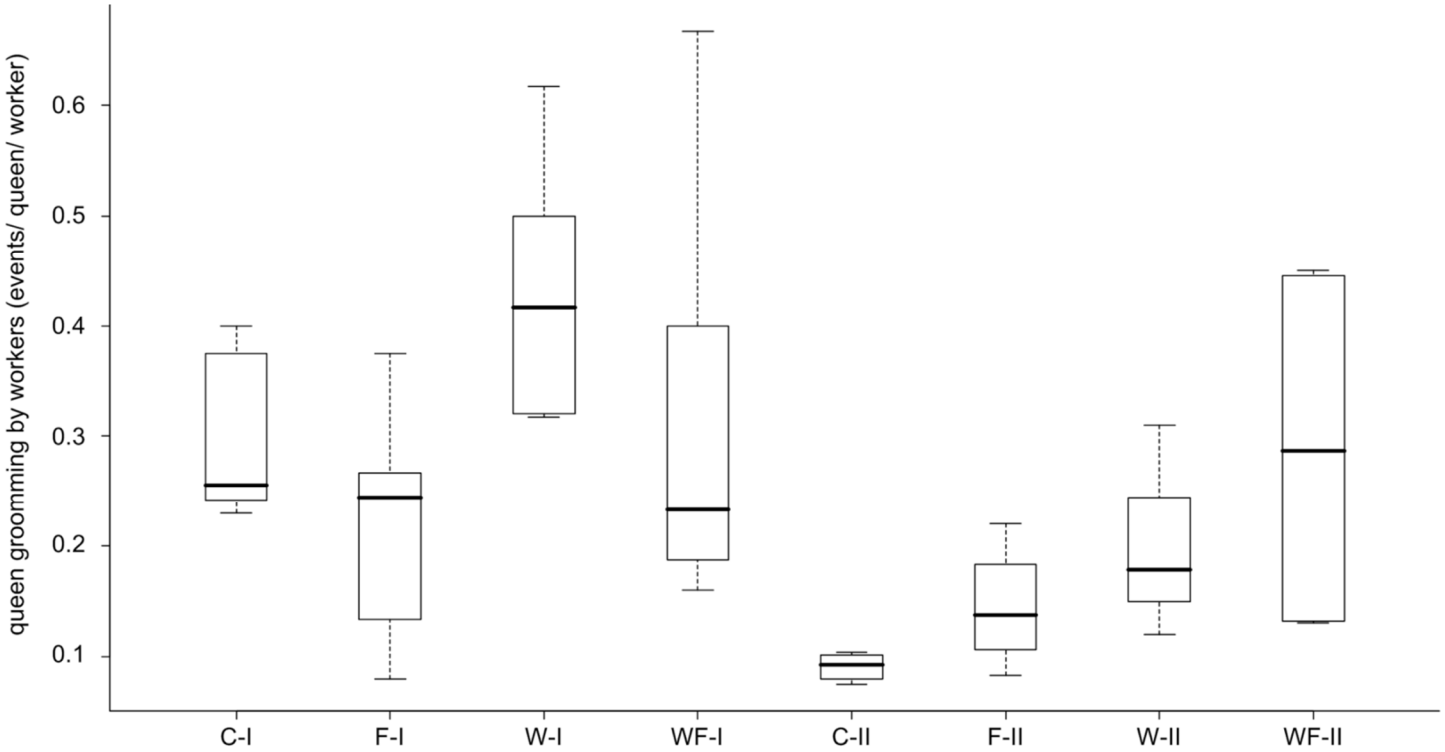

Figure S2.4 Frequency of queens groomed by workers (observations per queen and worker during the total observation period, median, quartiles, range) of the ant *Leptothorax acervorum* from the low-skew population in Nürnberger Reichswald. Individual colonies were subjected to different types of stress (food reduction F, worker reduction W, or both FW) or left unmanipulated (control C) in two different seasons (July, I, and September, II). Outliers are indicated as circles.

Table S2.1. Median queen – worker ratios for low-skew and high-skew populations of *Leptothorax acervorum*.

population	# colonies (n)[¶]	queens/ colony	workers/ colony	queen – worker ratio	social structure reproductive skew	reference
Germany	35	3.0 (2.5, 5.5)	86.0 (57.0, 120.0)	0.049 (0.030, 0.103)	facultative polygyny low	[1]
UK	5	8.0 (7.0, 11.0)	127.0 (95.0, 151.0)	0.070 (0.046, 0.084)	facultative polygyny low	[2]
UK (Santon) *	31	4.0 (3.0, 5.5)	62.0 (42.0, 121.5)	0.059 (0.041, 0.103)	facultative polygyny low	[3]
UK (Roydon)	25	3.0 (2.0, 7.0)	48.0 (25.0, 68.0)	0.095 (0.063, 0.135	facultative polygyny low	[3]
UK (Aberfoyle) *	13	10.0 (5.0, 20.0)	174.0 (68.0, 514.0)	0.077 (0.049, 0.095)	facultative polygyny low	[3]
Japan	4	5.5 (5.0, 6.3)	32.5 (27.5, 39.0)	0.192 (0.151, 0.223)	functional monogyny high	[4]
Spain *	50	14.5 (6.0, 21.8)	54.5 (32.0, 77.3)	0.219 (0.125, 0.337)	functional monogyny high	[5, 6]

Quartiles are given in parenthesis. * Populations were sampled during two different seasons. [¶] Only colonies with two or more queens (polygynous) were used in the analysis.

References

[1] Heinze J, Lipski N, Schlehmeyer K, Hölldobler B (1995) Colony structure and reproduction in the ant *Leptothorax acervorum*. Behav Ecol 6: 359-367.

[2] Bourke AFG (1991) Queen behaviour, reproduction and egg cannibalism in multiple- queen colonies of the ant *Leptothorax acervorum*. Anim Behav 42: 295-310.

[3] Chan GL, Hingle A, Bourke AFG (1999) Sex allocation in a facultatively polygynous ant: between-population and between-colony variation. Behav Ecol 10: 409-421.

[4] Ito F (2005) Mechanisms regulating functional monogyny in a Japanese population of *Leptothorax acervorum* (Hymenoptera, Formicidae): dominance hierarchy and preferential egg cannibalism. Belg J Zool 135: 3-8.

[5] Felke M, Buschinger A (1999) Social organization, reproductive behaviour and ecology of *Leptothorax acervorum* (Hymenoptera, Formicidae) from the Sierra de Albarracin in central Spain. Insectes Soc 46: 84-91.

[6] Felke M (1999) Biologie, soziogenetische Kolonie- und Populationsstruktur, sowie taxonomische Stellung einer zentralspanischen *Leptothorax acervorum*-Population (Hymenoptera; Formicidae). Doctoral thesis, Technische Universität Darmstadt.

Table S2.2 Number of inseminated queens and initial queens (in brackets) per replicate colony and treatment. Individual colonies were subjected to different types of stress (food reduction F, worker reduction W, or both FW) or left unmanipulated (control C).

replicate colonies	treatment C	F	W	FW
I	5 (6)	5 (6)	3 (3)	3 (3)
II	2 (3)	4 (4)	5 (6)	5 (6)
III	5 (5)	3 (3)	3 (4)	3 (3)
IV	3 (4)	3 (6)	2 (3)	4 (5)
V	3 (4)	7 (7)	3 (4)	2 (5)
VI	3 (3)	6 (6)	5 (6)	5 (5)
VII	5 (6)	5 (5)	1 (5)*	5 (7)
VIII	7 (7)	6 (6)	5 (7)	6 (6)
IX	5 (5)	6 (6)	7 (7)	5 (5)

* Colony was removed from analysis.

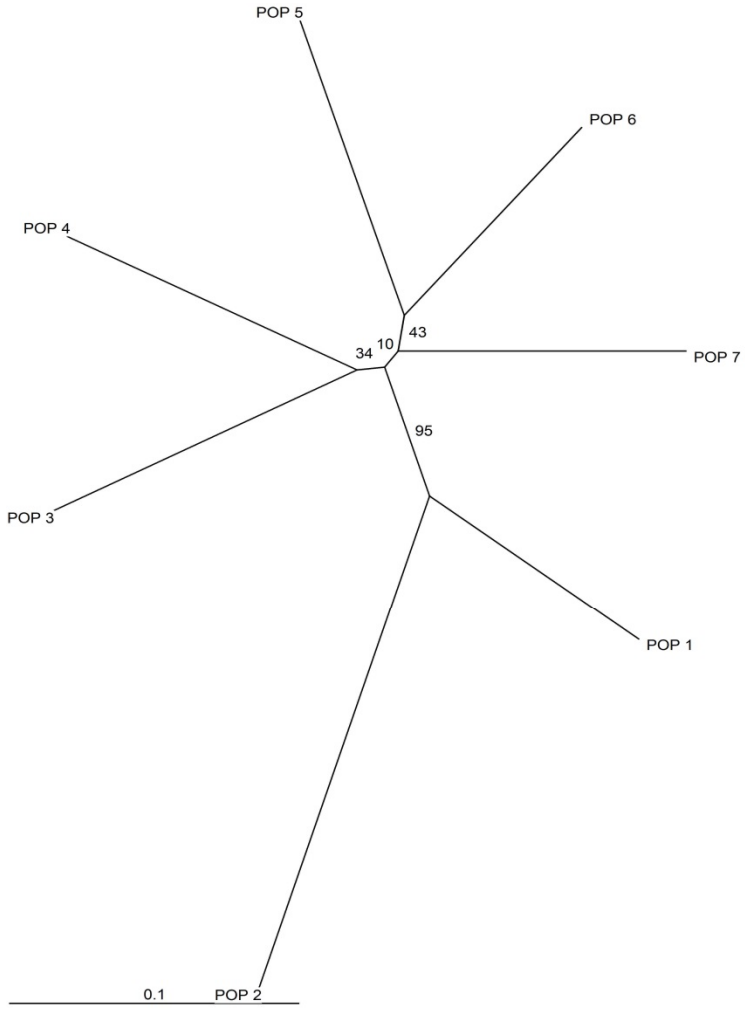

Figure S3.1 Unrooted neighbor-joining tree (for null allele corrected microsatellite data) using Nei's D_A distance, with bootstrap values given as numbers close to nodes. POP 1: *PY I*, POP 2: *PY II*, POP 3: *SNW I*, POP 4: *SNW II*, POP 5: *SG*, POP 6: *SA*, POP 7: *SD*. For population's ID and location see Table 3.1 and Figure 3.1.

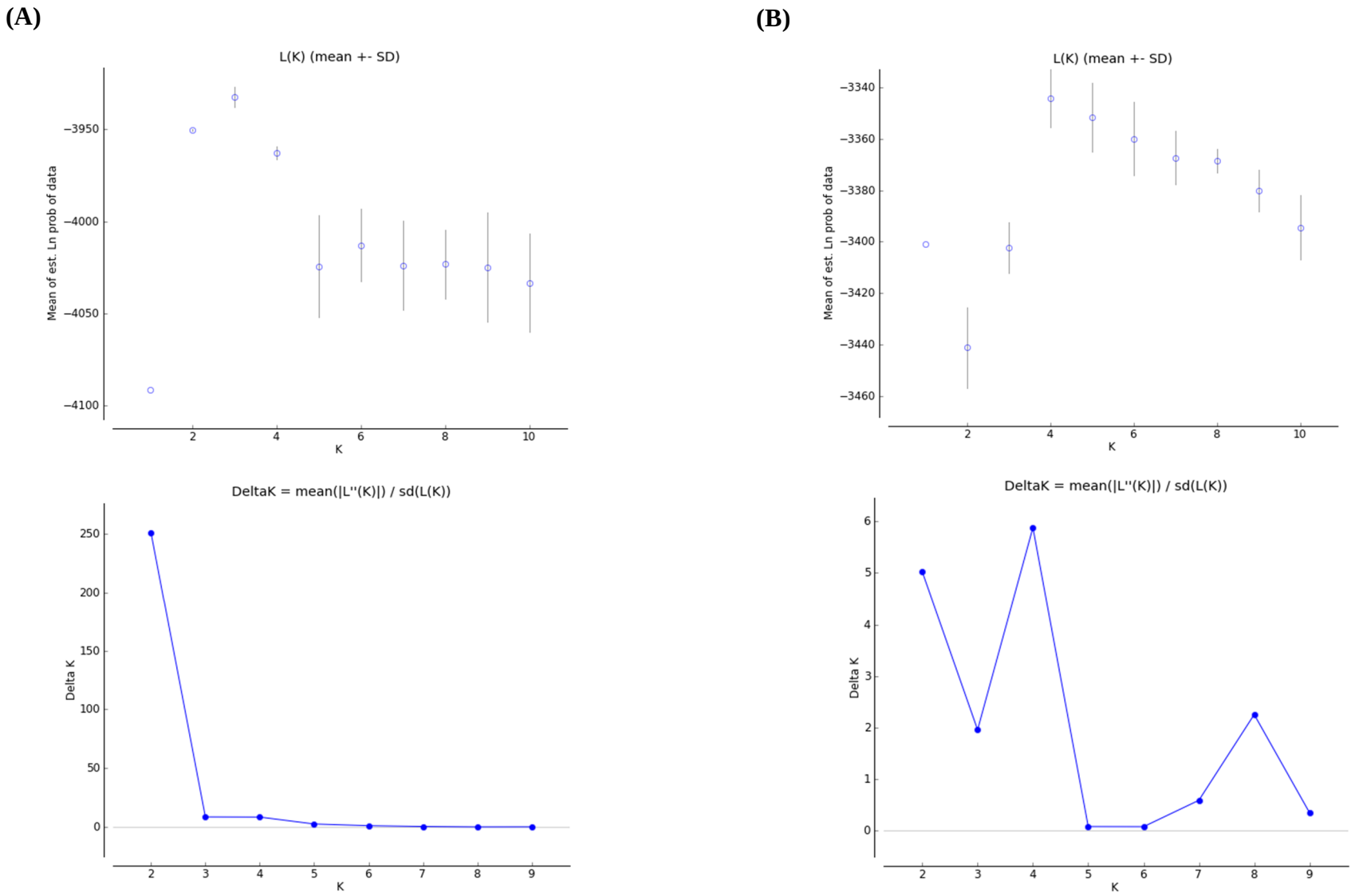

Figure S3.2 Estimated Ln probability of data and ΔK – values for (A) the 1st (all locations included) and (B) 2nd STRUCTURE analysis (PY II removed).

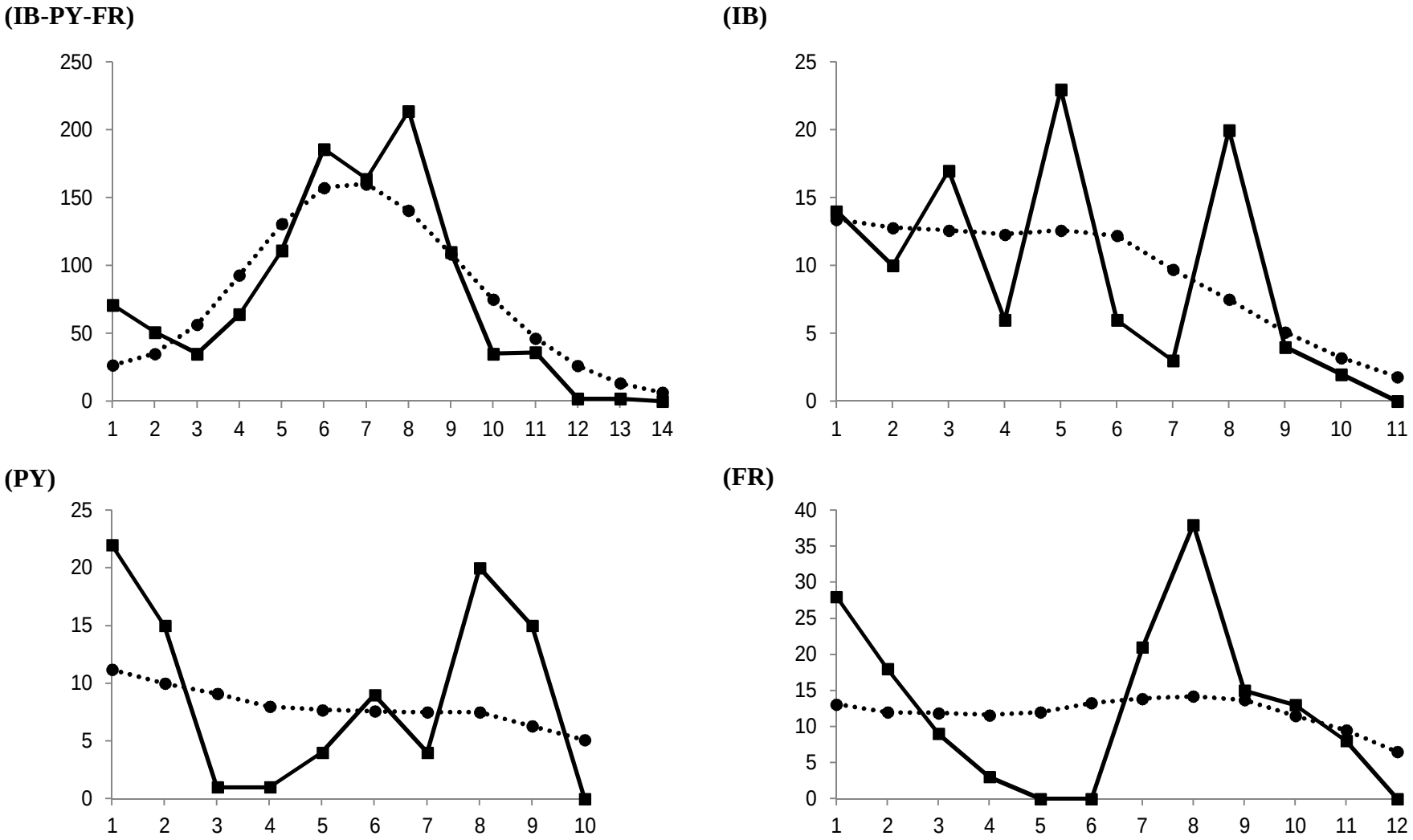

Figure S3.3 Distribution of pairwise differences between mtDNA haplotypes for the total dataset (IB-PY-FR), inner Iberia (IB), Pyrenees (PY) and France (FR). Solid line: Observed frequency, Dashed line: expected frequency (mean of 1000 replicates).

Table S3.1 Null Allele frequencies calculated with MICRO-CHECKER 2.2.3 using Brookfield method per locus and for each population. Populations: eastern Pyrenees (PY I), western Pyrenees (PY II), Cantabrian Mts. I (SNW I), Cantabrian Mts. II (SNW II), S^{re} de Gúdar (SG), S^{re} de Albarracin (SA) and S^{re} de la Demanda (SD).

Locus	Brookfield 1	*PY I*
GA1	0.085	
GA2	0.126	
GT223	0.056	
GT218	0.127	
GT1	0.073	
GT2	-0.029	
L18	0.001	
Myrt3	-0.081	
2MS67	0.193	
2MS46(II)	0.140	
		PY II
GA1	-0.113	
GA2	0.069	
GT223	-0.020	
GT218	-0.023	
GT1	-0.033	
GT2	0.023	
L18	-0.014	
Myrt3	0.050	
2MS67	0.045	
2MS46(II)	0.006	
		SNW I
GA1	-0.009	
GA2	0.074	
GT223	-0.049	
GT218	0.032	
GT1	0.028	
GT2	0.211	
L18	0.114	
Myrt3	0.020	
2MS67	0.164	
2MS46(II)	-0.046	
		SNW II
GA1	-0.054	
GA2	-0.017	
GT223	-0.095	
GT218	-0.056	
GT1	0.137	
GT2	0.105	
L18	-0.050	
Myrt3	-0.080	

Locus	Brookfield 1	*SNW II* cont.
2MS67	0.082	
2MS46(II)	-0.020	

Locus	Brookfield 1	*SG*
GA1	0.075	
GA2	-0.004	
GT223	0.065	
GT218	0.039	
GT1	0.135	
GT2	0.234	
L18	0.054	
Myrt3	-0.028	
2MS67	0.064	
2MS46(II)	0.046	
		SA
GA1	0.011	
GA2	-0.013	
GT223	-0.092	
GT218	0.243	
GT1	0.068	
GT2	0.101	
L18	-0.156	
Myrt3	0.041	
2MS67	0.065	
2MS46(II)	0.147	
		SD
GA1	0.112	
GA2	-0.021	
GT223	-0.041	
GT218	0.021	
GT1	0.136	
GT2	0.091	
L18	0.090	
Myrt3	-0.036	
2MS67	0.135	
2MS46(II)	-0.022	

Table S3.2 F_{ST} – values for Pyrenean and inner Iberian population pairs of *L. acervorum*, calculated from null allele corrected microsatellite data.

ID	PY I	PY II	SNW I	SNW II	SG	SA
PY II	0.072					
SNW I	0.072	0.121				
SNW II	0.062	0.119	0.085			
SG	0.038	0.127	0.082	0.068		
SA	0.050	0.115	0.048	0.076	0.047	
SD	0.056	0.101	0.041	0.089	0.057	0.019

For details on population ID and location see Table 3.1and Figure 3.1.

Table S3.3 Test of deviation from Hardy-Weinberg equilibrium (HWE) in microsatellite loci of *L. acervorum*.

popID	GA1	GA2	GT223	GT218	GT1	GT2	L18	Myrt3	2MS67	2MS46II
PY I	0.0653	0.0064	0.0153	0.1155	0.0022	0.7880	0.3220	0.9347	0.0065	0.0060
PY II	0.1096	0.0707	0.9008	0.4676	0.4946	0.5268	0.0433	0.0667	0.1050	0.2653
SNW I	0.7236	0.0290	0.8820	0.7046	0.0229	0.0012	0.0890	0.9079	0.0504	0.9895
SNW II	0.8213	0.6189	0.8817	0.2193	0.0371	0.4012	1.0000	0.4460	0.1585	0.7121
SG	0.3187	0.8237	0.4870	0.6041	0.0008	0.0062	0.6003	0.9193	0.0897	0.2941
SA	0.8506	0.8227	0.8385	**0.0003***	0.0262	0.3378	0.1442	0.3199	0.6040	0.0082
SD	0.0426	0.5906	0.1925	0.7484	**0.0000***	0.3919	0.2967	0.5947	0.0926	0.5423

Significant tests indicated by bold type and asterisk. Significance level adjusted for multiple tests by Bonferroni correction (n = 70): * α = 0.00071. For details on population ID see Figure 3.1 and Table 3.1.

Table S3.4 Comparative literature survey of various socially polymorphic Hymenoptera.

species	type of social polymorphism	genetic differentiation btw. forms		genetic basis	phenotypic plasticity	spatial distribution	reference
		mtDNA	ncDNA				
Solenopsis invicta	soc. structure (Q-no.)	yes	partial	gen diff, gec ream	no	sympatric	[1-3]
Formica selysi	soc. structure (Q-no.)	n/a	no	"social chromosome"	no	sympatric	[4-6]
Formica fusca	soc. structure (Q-no.)	n/a	no	suggested (field obs)	no	sympatric	[7]
Formica truncorum	soc. structure (Q-no.)	yes	yes	gen diff	no	sympatric	[8]
Veromessor pergandei	colony foundress (Q-no.)	n/a	no	suggested (lab obs)	n/a	para-/ allopatric	[9]
Pogonomyrmex californicus	colony foundress (Q-no.)	n/a	n/a	suggested (lab obs)	n/a	para-/ allopatric	[10]
Leptothorax acervorum	soc. structure (Q-no.)*	no	no	suggested (lab obs, HS)	yes (lab exp,LS)	para-/ allopatric	[11-13]
Halictus rubicundus (sweat bee)	solitary-social transition	no	no	no	yes (field exp)	para-/ allopatric	[14, 15]

* functional monogyny vs. facultative polygyny, gen diff: genetic differentiation, gec ream: genomic rearrangement, obs: observation, exp: experiment, HS & LS: high & low skew populations.

References

[1] Shoemaker DD, Ross KG (1996) Effects of social organization on gene flow in the fire ant *Solenopsis invicta*. Nature 383: 613-616.

[2] Gotzek D, Ross KG (2007) Genetic regulation of colony social organization in fire ants: an integrative overview. Q Rev Biol 82: 201-266.

[3] Wang J, Wurm Y, Nipitwattanaphon M, Riba-Grognuz O, Huang YC, Shoemaker D, Keller L (2013). A Y-like social chromosome causes alternative colony organization in fire ants. Nature 493: 664-668.

[4] Chapuisat M, Bocherens S, Rosset H (2004) Variable queen number in ant colonies: no impact on queen turnover, inbreeding, and population genetic differentiation in the ant *Formica selysi*. Evolution 58: 1064-1072.

[5] Purcell J, Chapuisat M (2013) Bidirectional shifts in colony queen number in a socially polymorphic ant population. Evolution 67: 1169-1180.

[6] Purcell J, Brelsford A, Wurm Y, Perrin N, Chapuisat M (2014) Convergent genetic architecture underlies social organization in ants. Current Biology 24: 2728-2732.

[7] Bargum K, Helanterä H, Sundström L (2007) Genetic population structure, queen supersedure and social polymorphism in a social Hymenoptera. J Evol Biol 20: 1351-1360.

[8] Gyllenstrand N, Seppä P, Pamilo P (2005) Restricted gene flow between two social forms in the ant *Formica truncorum*. J Evol Biol 18: 978-984.

[9] Helms KR, Helms Cahan S (2012) Large scale regional variation in cooperation and conflict among queens of the desert ant *Messor pergandei*. Anim Behav 84: 499-507.

[10] Overson R, Gadau J, Clark RM, Pratt SC, Fewell JH (2013) Behavioral transitions with the evolution of cooperative nest founding by harvester ant queens. Behav Ecol Sociobiol: DOI 10.1007/s00265-013-1618-2.

[11] Trettin J, Haubner M, Buschinger A, Heinze J (2011) Queen dominance and worker policing control reproduction in a threatened ant. BMC Ecology 11: 21.

[12] Trettin J, Seyferth T, Heinze J (2014) Behavioral Plasticity in Ant Queens: Environmental Manipulation Induces Aggression among Normally Peaceful Queens in the Socially Polymorphic Ant *Leptothorax acervorum*. PLoS ONE 9: e95153. doi:10.1371/journal.pone.0095153.

[13] Gill RJ, Arce A, Keller L, Hammond RL (2009) Polymorphic social organization in an ant. Proceedings of the Royal Society B 276: 4423-4431.

[14] Soro A, Field J, Bridge C, Cardinal SC, Paxton RJ (2010) Genetic differentiation across the social transition in a socially polymorphic sweat bee, *Halictus rubicundus*. Mol Ecol 19: 3351-3363.

[15] Field J, Paxton RJ, Soro A, Bridge C (2010) Cryptic plasticity underlies a major evolutionary transition. Curr Biol 20: 2028-2031.